DEPENDENCE OF METABOLIC RATE ON OXYGEN CONCENTRATION IN RHODOSPIRILLUM RUBRUM

BENJAMIN RANKIN PETRIE, Jr.

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IN RHODOSPIRILLUM RUBRUM

BENJAMIN RANKIN PETRIE, Jr.

DEPENDENCE OF METABOLIC RATE

ON

OXYGEN CONCENTRATION

IN

RHODOSPIRILLUM RUBRUM

by

Benjamin Rankin Petrie, Jr.

Commander, United States Naval Reserve

Submitted in partial fulfillment
of the requirements
for the degree of
MASTER OF SCIENCE
IN
PHYSICS

United States Naval Postgraduate School
Mongerey, California

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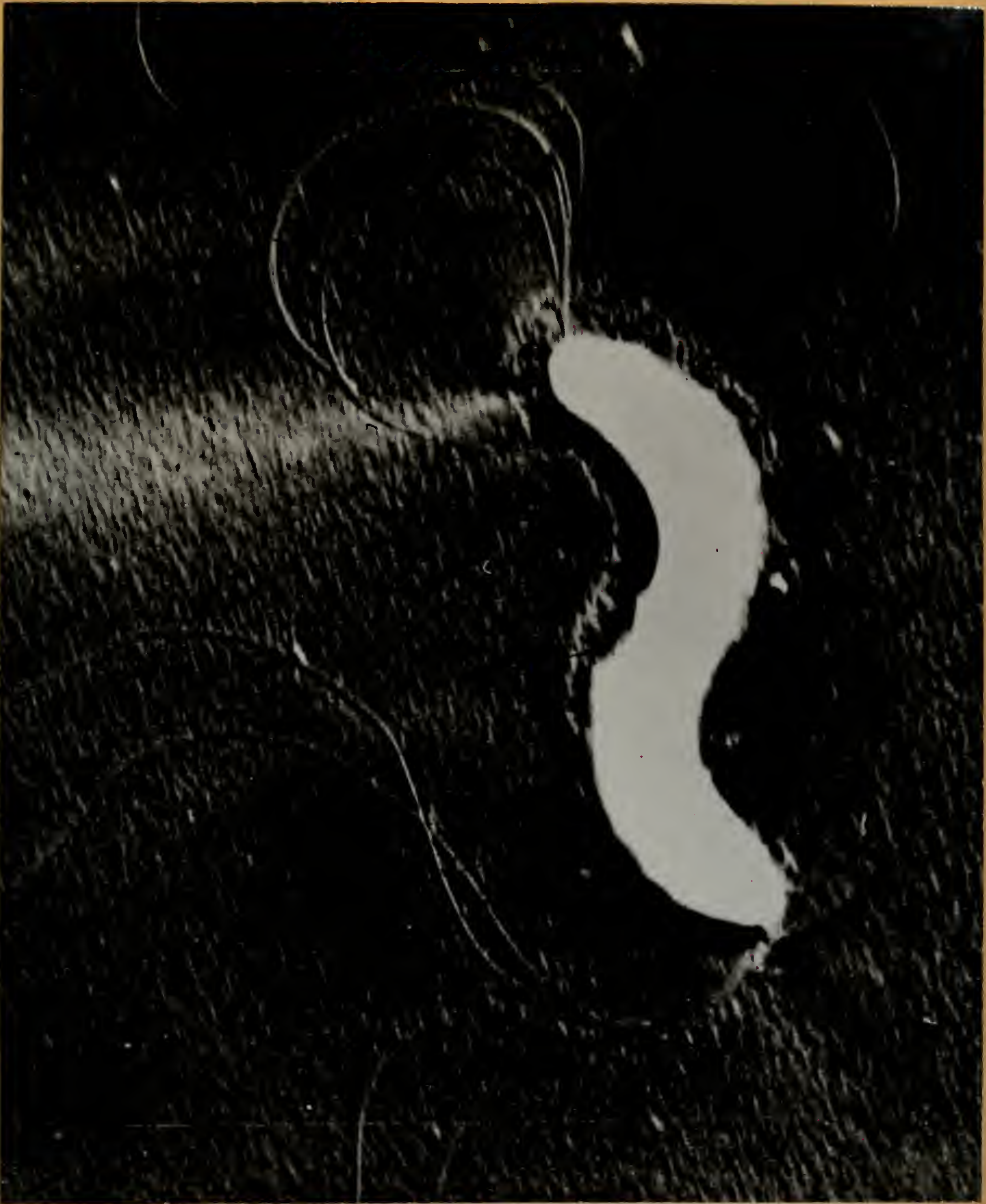
Thesis
P45



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Frontispiece

RHODOSPIRILLUM RUBRUM
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the thesis requirements for the degree of

MASTER OF SCIENCE

IN

PHYSICS

from the

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PREFACE

The work pursuant to this investigation was done in the Physics Laboratory of the U. S. Naval Postgraduate School, Monterey, California, in the Spring of 1954 for the purpose of lending assistance to the research studies of Dr. Clayton.

The writer wishes to thank his advisor, Dr. Roderick K. Clayton, for his suggestions, encouragement, and editorial advice; his absorbing interest in the Rhodospirillum rubrum and the problems attendant to the study of their metabolic behaviour provided a source of inspiration.

Appreciation is due Professor Sidney H. Kalmbach for his encouragement, to Dr. Hardin B. Jones of the University of California for consenting to be the "second reader" of this paper, to Mr. Milton K. Andrews, OMC Robert C. Moeller, and Mr. Thomas Burson for their aid in obtaining necessary equipment, and to Mrs. Lee Thorne and Mrs. Jerre' Ramlow for their help in preparing the manuscript.

The writer wishes to thank his wife, Jean, for her material assistance with this thesis, but whose patience and tolerance played an even more important role in its completion.

TABLE OF CONTENTS

	Page
CERTIFICATE OF APPROVAL	i
PREFACE	ii
TABLE OF CONTENTS	iii
LIST OF ILLUSTRATIONS	iv
TABLE OF SYMBOLS & ABBREVIATIONS	v

CHAPTER

I. INTRODUCTION	1
II. INSTRUMENTATION	23
III. PROCEDURE	28
IV. DISCUSSION OF RESULTS	34
APPENDIX A: THE WARBURG ANALYSIS	37
APPENDIX B: THE FLOW METER	40
APPENDIX C: THE DENSITOMETER	47
APPENDIX D: THE pH METER	50
APPENDIX E: GLOSSARY OF TERMS	51
APPENDIX F: SAMPLE RUN DATA	56
BIBLIOGRAPHY	62

LIST OF ILLUSTRATIONS

Page

Rhodospirillum rubrum (x 25,000) - - Frontispiece

1. Graphs of the rate of substrate decomposition versus per cent air by Rhodospirillum rubrum under various light intensities 14
2. Graphs of the rate of carbon assimilation versus per cent air by Rhodospirillum rubrum under various light intensities 16-17
3. Apparatus Binder pocket
4. Plot of sample run Binder pocket
5. Aerotactic behaviour of Rhodospirillum rubrum under various light intensities 20
6. Plot of rate/maximum rate of substrate decomposition (as measured by volume of 0.0417 N HCl added to maintain pH 6.80) versus per cent air/"saturating" per cent air 36
7. The flowmeter 44
8. Bacterial densitometer 48
9. Bacterial densitometer chart 49
10. The pH meter 50

TABLE OF SYMBOLS & ABBREVIATIONS

Rh. rubrum	Rhodospirillum rubrum
ca.	circa; approximately; about
N	Normal*
M	Molar*
ml.	milliliter (L^3)
l.	liter (L^3)
cm.	centimeter (L)
r	density (M/L^3)
h	elevation of center of gravity of gas volume above some arbitrary datum line (L)
Q	flow rate (L^3/T)
P	pressure (M/T^2L)
V	velocity (L/T)
K	flow coefficient of pressure ($L^{1/2}/M^{1/2}$)
g	acceleration due to gravity (L/T^2)
mμ	millimicron; 1×10^{-9} meters

*See Appendix E

CHAPTER I

INTRODUCTION

Rhodospirillum rubrum, a bacterium which is illustrated in the electron micro-photograph on the frontispiece, is a member of Class Schizomycetes, Order Eubacteriales, Suborder Rhodobacteriineae, Family Athiorhodaceae (1)*. These bacteria are lophotricate, rigid, spiral, non-spore forming, and gram-negative micro-organisms. In the absence of light Rh. rubrum are heterotrophic aerobes, but in the presence of sufficient illumination are photosynthetic anaerobes; it is this characteristic which forms the basis for their study, as will be discussed in some detail subsequently. These bacteria have the ability to grow in a wide variety of media, often exhibiting striking morphological variations (for the same strain) attributable to environmental conditions, with a three-fold variation in width (0.5 to 1.5 microns) and even more pronounced variations in length (from 2 to 50 microns); indeed, in the same cultures there are considerable differences among individual bacteria, probably due to changes in the medium with progressive development of the mocro-organisms(2).

Rhodospirillum rubrum are commonly referred to as purple bacteria, but, like their morphology, their color is environment dependent, ranging from dark purple to pink; aerobic development inhibits their pigment production.

The optimum temperature for their growth ranges between 30° and 37°C.; the pH of the culture medium may vary from 6.0 to 8.5, with some inhibition of growth occurring in the most acid medium (2).

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*This classification varies somewhat among microbiologists.

It has been shown that these bacteria require d-glutamic acid, d-arginine, cystine, and biotin for growth in a chemically defined medium (3).

Rh. rubrum apparently do not produce proteolytic enzymes; at least, gelatin is not liquified by any of the strains and it can be demonstrated that the ammonium salts in the medium constitute the main nitrogen source (2).

The Family Athiordaceae are widespread in nature, being present in the muds and surface water of nearly all fresh water bodies, particularly in stagnant pools; they are sometimes grown from sand and soil samples, but their natural environment seems to be an aqueous one (2). They are of no economic interest at present (4).

The immediate purpose of the investigation is implicit in the title of this paper, with the work being done as a component of Dr. R. K. Clayton's studies on the metabolic rates of the Rh. rubrum under a variety of light intensities and oxygen concentrations (most of which can and has been done by employing the Warburg Gas Analysis technique*). Before stating the underlying motivation, however, it would be well to review some of the unique characteristics of Rb. rubrum and other background material.

It has been more than one hundred and twenty five years since the German chemist Wöhler invalidated the "vital force" theory by his synthesis of the living cell product, urea, from the inorganic ammonium cyanate (5)**. It may well be -- and it is so expected -- that one hundred and twenty-five years hence much of the mystery surrounding life processes will also be stripped away, as was the belief of Wöhler's day that only living cells could produce "organic" materials. Today one of the least understood properties of the living cell is

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*See Appendix A

** See also any elementary organic chemistry text

irritability (response to stimulus). The attack on this field has been launched from many fronts; physiologists, psychologists, physicists, chemists, biologists, philosophers, and others are concerning themselves with this problem.

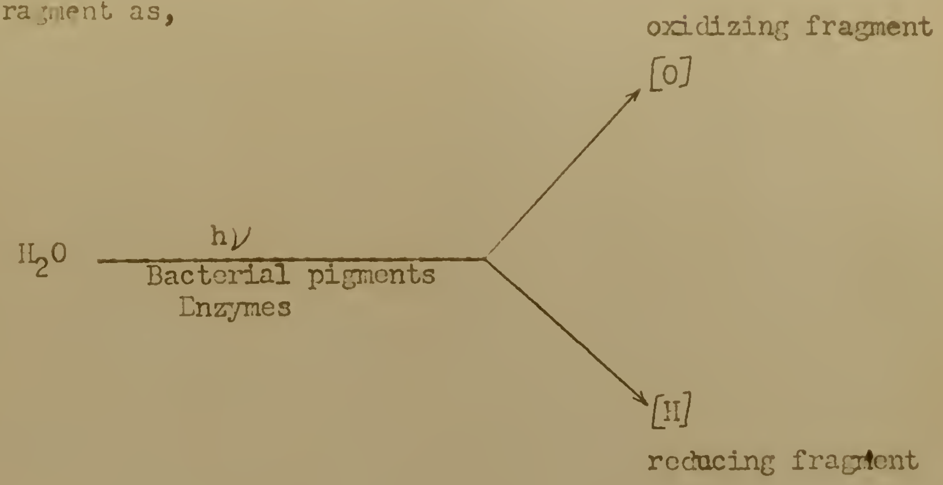
It may be argued that already it has been demonstrated that irritability is not peculiar to a living cell. Certainly a Geiger counter is "irritable"; it responds to a stimulus (an ionizing event), its response is "all-or-none", it has a refractory period (dead time), and it may even be said to exhibit the phenomenon of tetany (when ionizing events are occurring so rapidly that the mechanical counter is jammed, or when the voltage across the counter is raised to the area of continuous discharge). Possibly even more striking is Dr. Ralph Lillie's iron-wire model of a nerve, discussed in physiology texts(6), which exhibits an all-or-none response to both mechanical and electrical stimuli, and the phenomena of action potential, refractory period, and electro-tonus.

Yet, analogous as these non-living systems are to the living, the analogies quickly break down on extension; so we have in no way duplicated the irritability of the living cell, but merely imitated it. More to the point, we have gained no insight as to how the cell responds, or to the detailed nature of the phenomenon of irritability.

Turning our attention now to the Rh. rubrum, their ability to adapt either to an aerobic or anaerobic metabolism holds considerable promise as an approach to an understanding of the sensitivity peculiar to living systems.

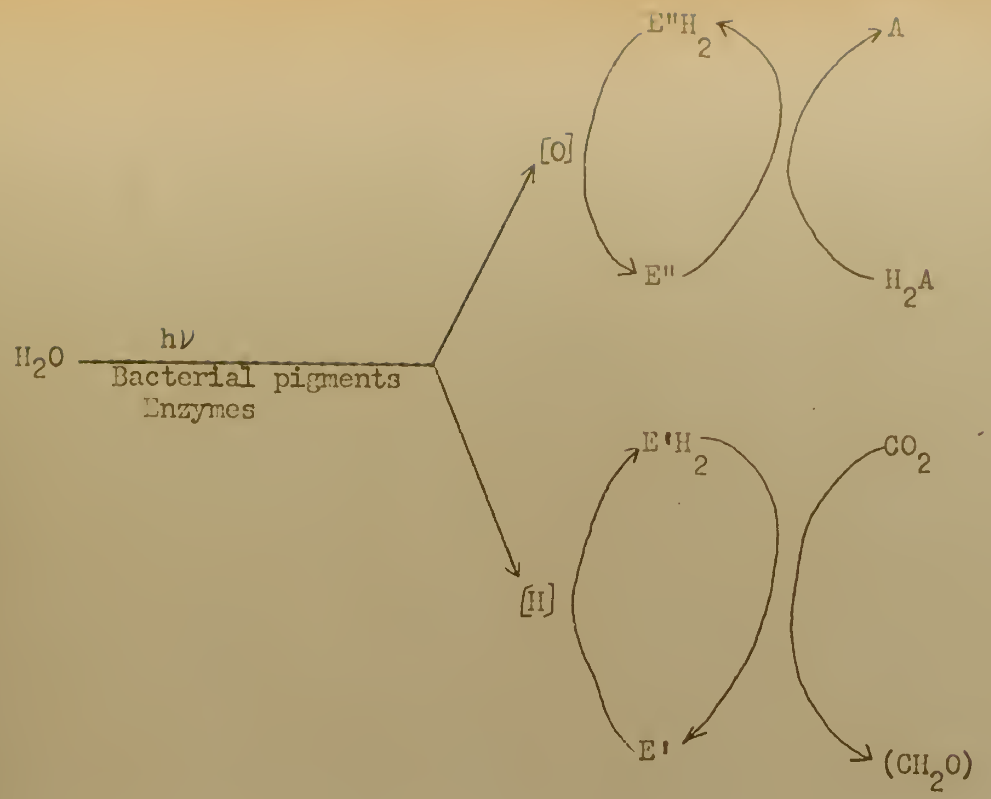
Van Niel (7) gives us an insight into this in his comparison of

the photosynthetic and chemosynthetic processes. The evidence is now conclusive that in photosynthesis in green plants it is the water, and not the CO_2, which is broken down to form O_2. In bacterial photosynthesis, however, no oxygen is formed. We assume that in bacterial photosynthesis water is "split" into an oxidizing fragment and a reducing fragment as,



The reducing fragment brings about the reduction of carbon dioxide to cell materials, i.e., $CO_2 \xrightarrow{[H]} (CH_2O)$ where (CH_2O) merely represents, after the convention adopted by van Niel (7) and others, the reduction product of CO_2, "in general, 'organic matter'." The oxidizing fragment, however, must be removed from the reaction vicinity; failure to do so will result in stopping the reactions, probably by some means such as poisoning the bacterial photosynthetic pigment or the enzyme involved, or through the occurrence of a back reaction with the reducing fragment.

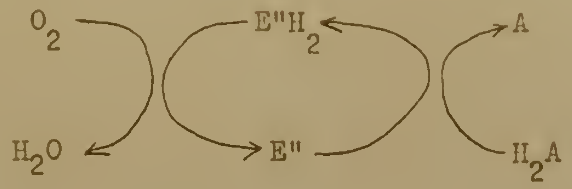
It appears that the last steps are also mediated by some enzyme system, and that the photosynthetic process can be illustrated as follows:

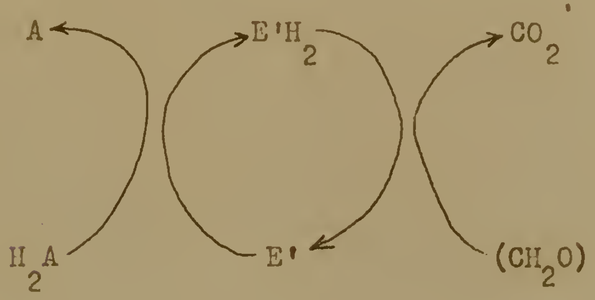


Here E is generic for enzyme system, with E" referring to the system responsible for acting as the hydrogen donor in reducing the oxidizing fragment and as the hydrogen acceptor in oxidizing the substrate; E' indicates the enzyme system responsible for the reduction of CO_2, but which is first reduced itself by the reducing fragment [H] from the water. (In green plant photosynthesis, the enzyme corresponding to E" can evolve oxygen and reform itself in the reduced state $E''H_2$.) H_2A is the substrate and serves as the hydrogen donor.

Now, in the absence of light, Rh. rubrum are able to carry out a typically oxidative metabolism. From the work of Blackman and Matthaei (demonstrating "that photosynthesis is the net result of a combination of photochemical ('light') and non-photochemical ('dark') reactions") and the comparison of the photosynthetic and oxidative equations for

the bacterial utilization of the same organic substrate, van Niel (7) concludes that the "degradation of the oxidizable substrate, whether in photosynthesis or in dark metabolism, must be accomplished by the same enzyme systems." Accepting this, then, and diagramming a scheme similar to the one shown for photosynthesis, we have for oxidative metabolism, where E" and E' are identical with those enzyme systems previously identified.





In this instance the molecular oxygen serves to oxidize $E''H_2$ to E", which in turn serves to oxidize the substrate H_2A. This oxidation of the substrate serves as a source of energy (in place of $h\nu$ in photosynthesis) which can be utilized to reduce the CO_2 to cell materials. It must be admitted that this is to some degree speculative, but the evidence indicates that the mechanism, if not actually the one pictured, is at least very similar to it.

It is to be noted that, insofar as utilization of the substrate is concerned, the photosynthetic metabolic path can be twice as

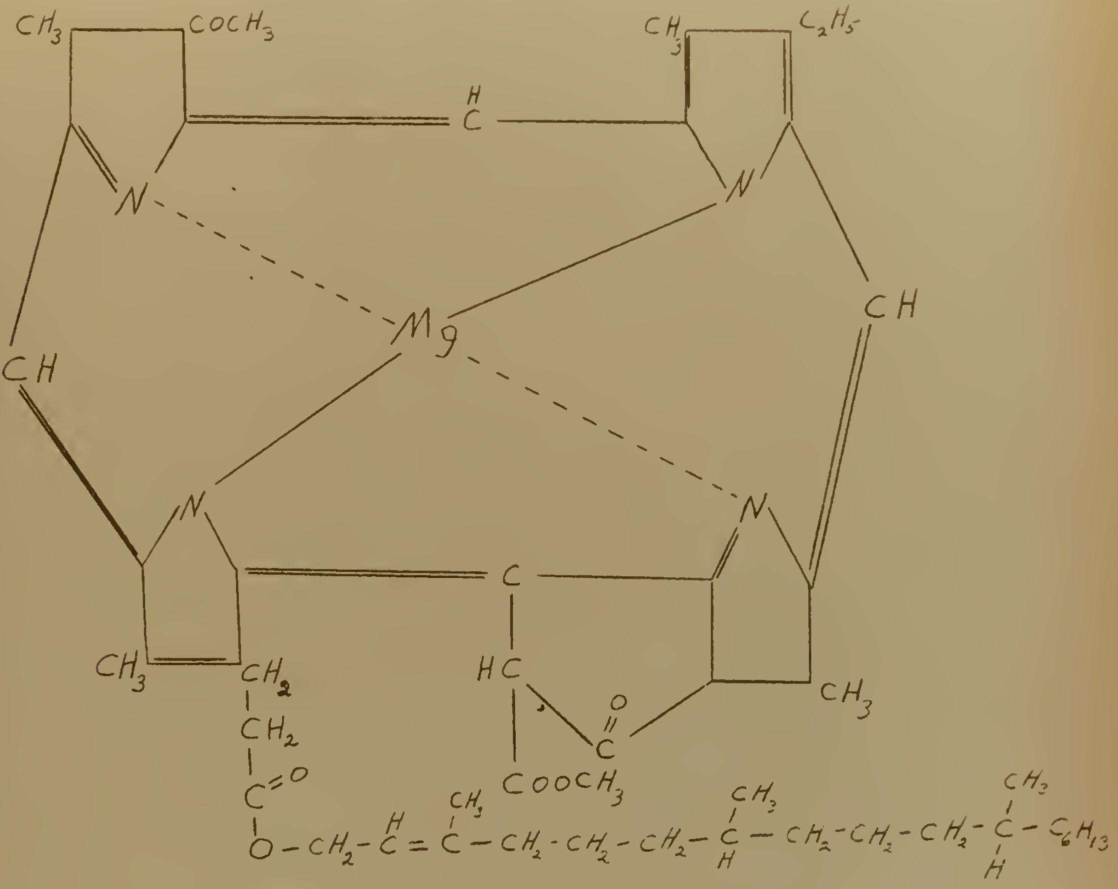
efficient in the production of cell materials. In the scheme we have represented, for photosynthesis one mole of CO_2 is reduced (and assimilated) per mole of substrate oxidized, while for the oxidate route one mole of CO_2 is reduced (and assimilated) per two moles of substrate oxidized. The importance of this will be discussed subsequently.

Giving attention now to the tactic behaviour of Rh. rubrum: It has long been known that these bacteria exhibit a positive phototaxis; i.e., that they congregate in a spot of light. Clayton (8) describes this phototactic response as "a reversal of swimming direction evoked by an abrupt decrease in the intensity of the light impinging on the bacterium", and credits Molisch with having observed the phenomenon of accomodation in this response; i.e., "the effectiveness of a decrease in illumination in evoking a response becomes greater as the decrease is made more abrupt".

Not so easily observed is the aerotactic behaviour. In the dark the bacteria show a positive aerotaxis by gathering in areas of high oxygen concentration just as they gather in areas of high light intensity; as they start to swim out of the area of high oxygen concentration (in the dark) they reverse their swimming direction and return. In the light, however, the response is just the opposite. They avoid areas of high oxygen concentration by reversing their swimming direction as they enter the area of increasing oxygen. Thus they show a positive phototaxis, a positive aerotaxis in the dark, and a negative aerotaxis in the light.

Since the pigments determine the wave length of the light absorbed, and this in turn determines the energy per mole-quantum received by the (photosynthesizing) organism, it is appropriate to give

some attention to the bacterial pigments of Rh. rubrum. There are two principal groups of bacterial pigments: (a) Those whose structure is related to chlorophyll (bacteriochlorophyll), one such substance having been reported to have the following structure (9):



Bacteriochlorophyll a ($C_{55}H_{72}N_4O_5Mg$)

and (b) those whose structures classify them as carotenoid pigments. Clayton (8) found for bacteriochlorophyll in the intact cells "a pronounced absorption maxima at 875 mμ and smaller maxima and 800 and 590 mμ ". The major carotenoid pigment in Rh. Rubrum is spirilloxanthin, $C_{48}H_{66}O_3$ (9), with absorption maxima at ca. 550, 510, and 480 mμ in the intact cells (where, as with bacteriochlorophyll, the

carotenoids are conjugated with protein). The absorption spectra of the intact cells do not show the maxima in the blue and green for the scarcer carotenoids since these are "masked by the maxima of the preponderant spirilloxanthin and by the effect of strong scattering" (8).

In tabulating the maxima in absorption spectra and in action spectra for photosynthesis and phototaxis of the Rh. rubrum, Clayton (8) showed that there were areas of disagreement among previous investigators, except for the photosynthetic and phototactic effectiveness peaks at the bacteriochlorophyll absorption maxima.

In view of the discordance mentioned, Clayton (8) re-examined the phototactic action spectrum using monochromatic light of greater spectral purity and also re-investigated the photosynthetic growth of Rh. rubrum in light absorbed solely by the carotenoid pigments, here using a light of greater intensity than that previously employed. In the latter instance it was found that the light of the wave lengths absorbed "is as effective for growth as it is for phototaxis, when compared to with the light absorbed by chlorophyll" (8).

In the re-examination of the phototactic action spectrum, the over-all result indicated that in general the maxima in the absorption spectrum correspond to maxima in the action spectra of both phototaxis and photosynthesis; exceptions which occur can be justifiably attributed to masking; i.e., it is indicated that although spirilloxanthin is phototactically active, one of the relatively scarce carotenoid pigments is considerably more active in proportion to its concentration in the bacteria, thus resulting in a masking of the scarcer carotenoid in the absorption spectrum and some masking of the phototactic action of the spirilloxanthin in the action spectrum.

To summarize, it is strongly indicated that there is a correspondence between absorption, photosynthetic activity, and phototactic response throughout the spectrum.

Based principally on the foregoing considerations, Manten (10) has hypothesized that a decrease in the rate of photosynthetic metabolism in the Rh. rubrum is responsible for a phototactic response. Clayton's (11) subsequent investigations have supported this hypothesis; he points out that, since accommodation is a characteristic of phototaxis, it is not likely that the "phototactic response depends simply upon a certain change in the 'steady state' rate of photosynthesis". This accommodation is explained by supposing that the phototactic response depends not "simply upon a -- change in the 'steady state' rate of photosynthesis" but rather "is associated with a transient disturbance in the photosynthetic process, and that this transient fluctuation must reach some threshold magnitude to evoke a response". Further, that "such a transient effect can occur at light intensities higher than that which corresponds to saturation for photosynthesis, as based on measurements of the steady rate". Manten's hypothesis thus interpreted " - - does not require that saturating light intensities for phototaxis and for photosynthesis be equal" (11).

It was found, on investigating the saturation intensities for photosynthesis and phototaxis, that the saturation intensity for phototaxis is either "roughly equal to or much greater than that for photosynthesis", which supports the explanation of accommodation given earlier. Additionally, "that the saturating intensity for phototaxis is influenced markedly by the nature of the substrate which

serves for photosynthesis" (11) lending further support to the premise of close relationship between photosynthesis and phototaxis.

With the preceding paragraphs in mind, it may be seen that there is evidence that Manten's hypothesis can be extended to include metabolic rate as a whole (oxidative plus photosynthetic) and all tactic responses (to light, to oxygen, etc.). This "Manten-Clayton" hypothesis would state that all tactic responses in Rh. rubrum are associated with a decrease in metabolic rate.

Finally, to formulate that at which we have previously hinted: The purpose of this whole pattern of investigations has been to gain a better understanding of the underlying processes in living cell irritability. It may be argued that, though an insight into the irritability of these micro-organisms might be forthcoming, this is no more reason for believing that from such a basis the physiological nerve fiber action, say, can be explained any more satisfactorily than by Lillie's iron-wire model to which reference was made earlier (let it be noted that there is no intention here to discount the value of such a model in arriving at a mechanistic understanding of irritability). This contention is not without some validity, but hardly constitutes a serious argument insofar as the possible worth of the investigations are concerned. First, in common with other living (and, in part, certain non-living) irritable systems, Rh. rubrum exhibit the following:* All-or-none response, refractory period, accomodation, summation, rythmicity, (12)(13) and to some degree Weber Law adherence (6). Second, the physiological pathways of the responses in the Rhodospirillum rubrum will have certain

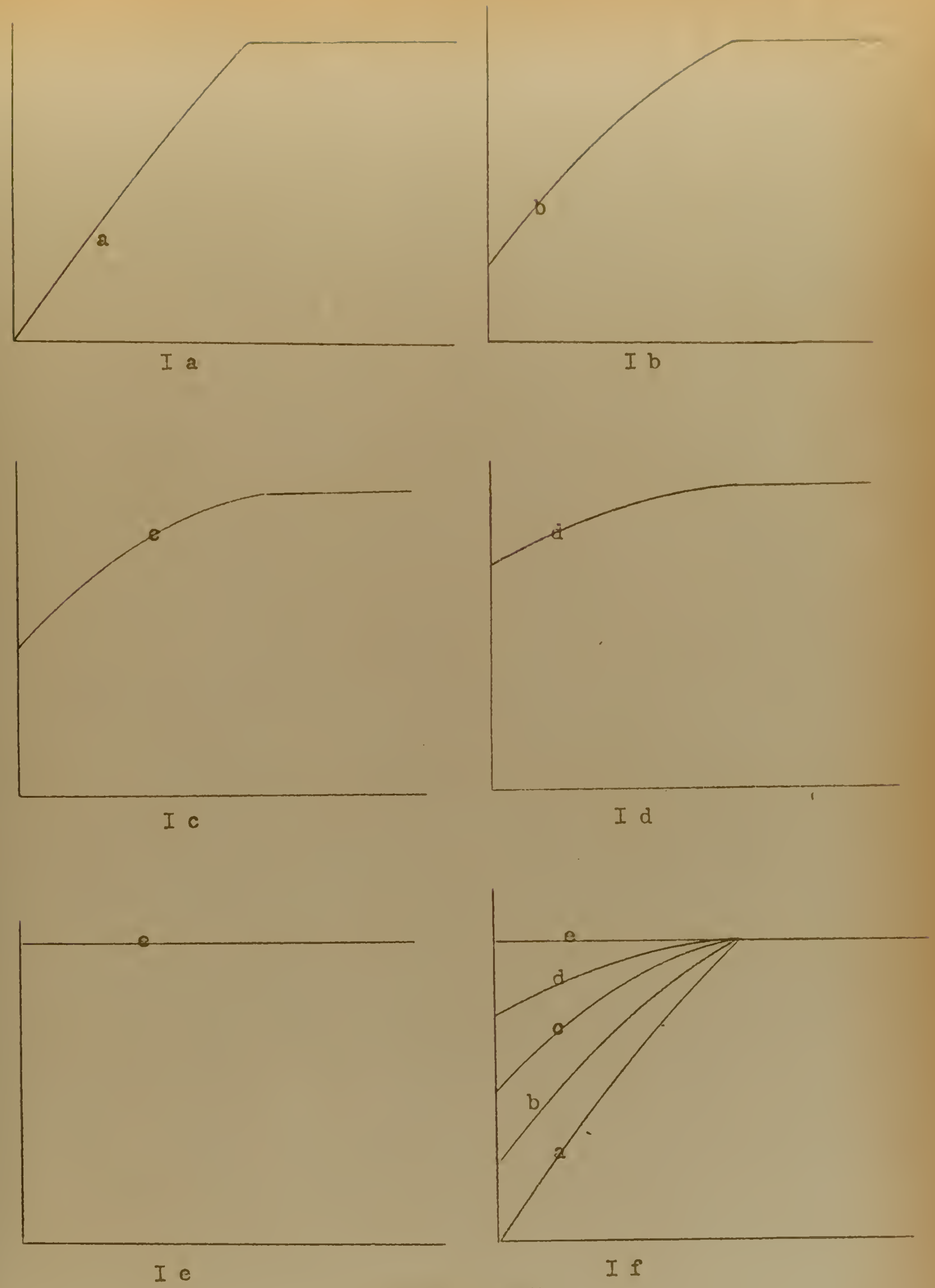
*See Appendix E (Glossary)

homologous as well as analogous relationships to the responses in other living tissue. At least, the responses of these micro-organisms are in no sense artificial, and it will doubtless be possible to test for homologies in other living systems.

Now, from what has been said about the photosynthetic and oxidative metabolic pathways, the phototactic and the aerotactic responses, and the similarity of the action spectra of phototaxis and photosynthesis in Rh. rubrum, a means for integration of all this into a method for accomplishing the previously mentioned purpose can be proposed. It is supposed that, when there occurs a decrease in the metabolic rate of the Rh. rubrum, there is a transient accumulation of some intermediary (in the metabolic path) which acts as a trigger for subsequent reactions which lead to a tactic response. By determining through which branch (the oxidative or the reductive) of the metabolic pathway the tactic response is mediated, i.e., in which branch the intermediate -- if such there be -- builds up, there will be obtained an insight as to what chemical processes are important for the tactic response, and these can be studied further; possibly even the triggering intermediate (or the taxis initiator, whatever it be) can be identified. Therefore, the first step will be to locate the "taxis trigger" in one or the other of the two branch processes, i.e., the oxidation of the substrate (as a source of energy and CO_2) or the reduction of the CO_2 (to cell material). Recalling the comparison made of the (possible) difference between the efficiency of photosynthetic and oxidative metabolism in substrate utilization (conversion of CO_2 into cell materials), we obtain a clue to how this may be accomplished. First, it will be necessary to observe

the phototactic and aerotactic responses under different light intensities and in varying concentrations of air; second, to determine the rate of substrate decomposition (not to be confused with substrate utilization by incorporation into cell materials) and of carbon assimilation under these different light and air environments. To see how the information thus obtained could be applied to let us make a few assumptions (in support of which there is some experimental evidence (14)) and follow a hypothetical set of data to its logical conclusion.

To begin with, let us suppose that the maximum attainable rate of substrate decomposition is the same for both photosynthetic and oxidative metabolism. Next, let us chart the rate of substrate decomposition versus the air concentration on a series of plots using the light intensity as our variable parameter. In the total absence of light, a curve (the curve determined in this investigation) of the type shown in Illustration I a is obtained, where the rate of substrate decomposition increases steadily up to its maximum, i.e., up to the point where the bacteria are receiving air as fast as they can utilize it. Where the intensity of illumination is such that the bacteria in complete absence of air are metabolizing at a rate corresponding to one-forth of the maximum, we obtain a curve as seen in Illustration I b. Similarly, curves are obtained for intensities of illumination corresponding to metabolic rates of one-half and three-fourths maximum, respectively, as shown in Illustrations I c and I d. As we increase the percent air available to the micro-organisms, some of the photosynthetic metabolism is replaced by the oxidative, but by the time the system (bacterial suspension) has been saturated with air, the maximum



I a I b I c I d I e I f

ILLUSTRATION I

Abscissas: Per cent air concentration

Ordinates: Rate of substrate decomposition

metabolic rate has been reached.

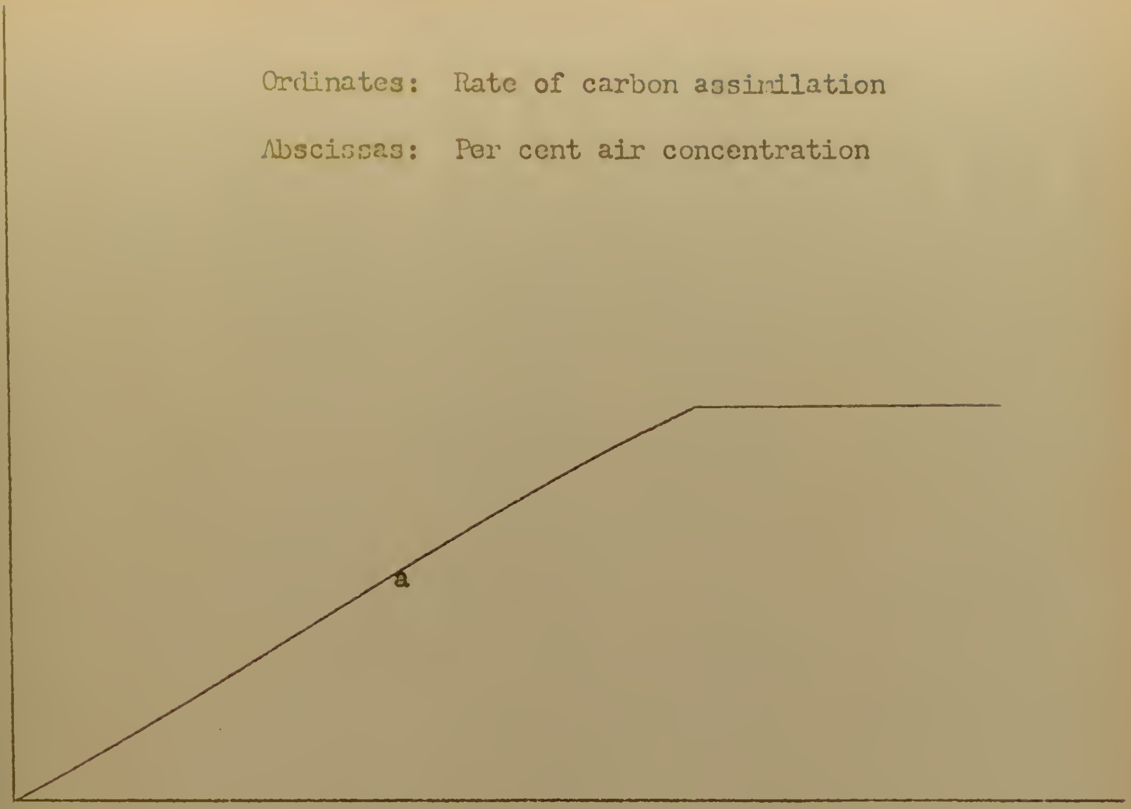
If, however, the incident illumination is of an intensity such that even in the absence of air the maximum rate of substrate decomposition is attained, the plot consists of the line denoting the maximum rate all the way across, as seen in Illustration I e. The result of superimposing all these curves on one plot is shown in Illustration I f.

Turning our attention now to the carbon assimilation rate (rate of $CO_2 \xrightarrow{[H]} (CH_2O)$ conversion), we will suppose -- as was indicated by comparison of the schemes presented earlier for photosynthetic and oxidative metabolism, and for which there is (as yet) fragmentary evidence (14) -- that when the Rh. rubrum are performing oxidative metabolism, one-half of the substrate is simply utilized as an energy source and is otherwise wasted. In other words -- to repeat-- with oxidative metabolism a mole of carbon is assimilated for every two moles of substrate oxidized, and with photosynthesis a mole of carbon is assimilated for every one mole of substrate oxidized. This means, then, that if the maximum rates of substrate oxidation for both types of metabolism are equal, the maximum rate of carbon assimilation would be twice as great with photosynthesis because, with respect to substrate, it is twice as efficient. To illustrate this, let us chart the rate of carbon assimilation versus the air concentration again using light intensity as a variable parameter. In the dark the curve will be identical with the curve shown in Illustration I a since nothing but oxidative metabolism occurs throughout the plot; this curve is represented in Illustration II a. Examining the other parametric extreme next, we would find that for light intense enough to produce

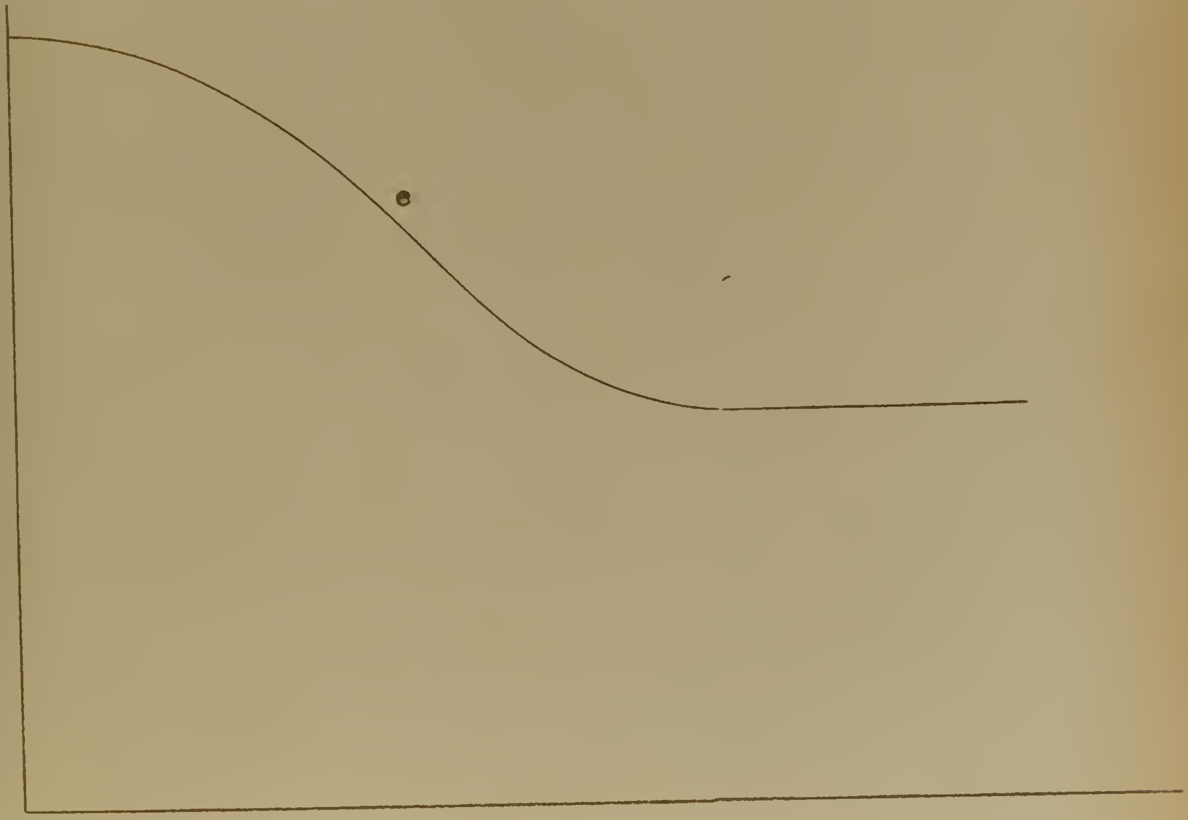
ILLUSTRATION II
(Part 1)

Ordinates: Rate of carbon assimilation

Abscissas: Per cent air concentration

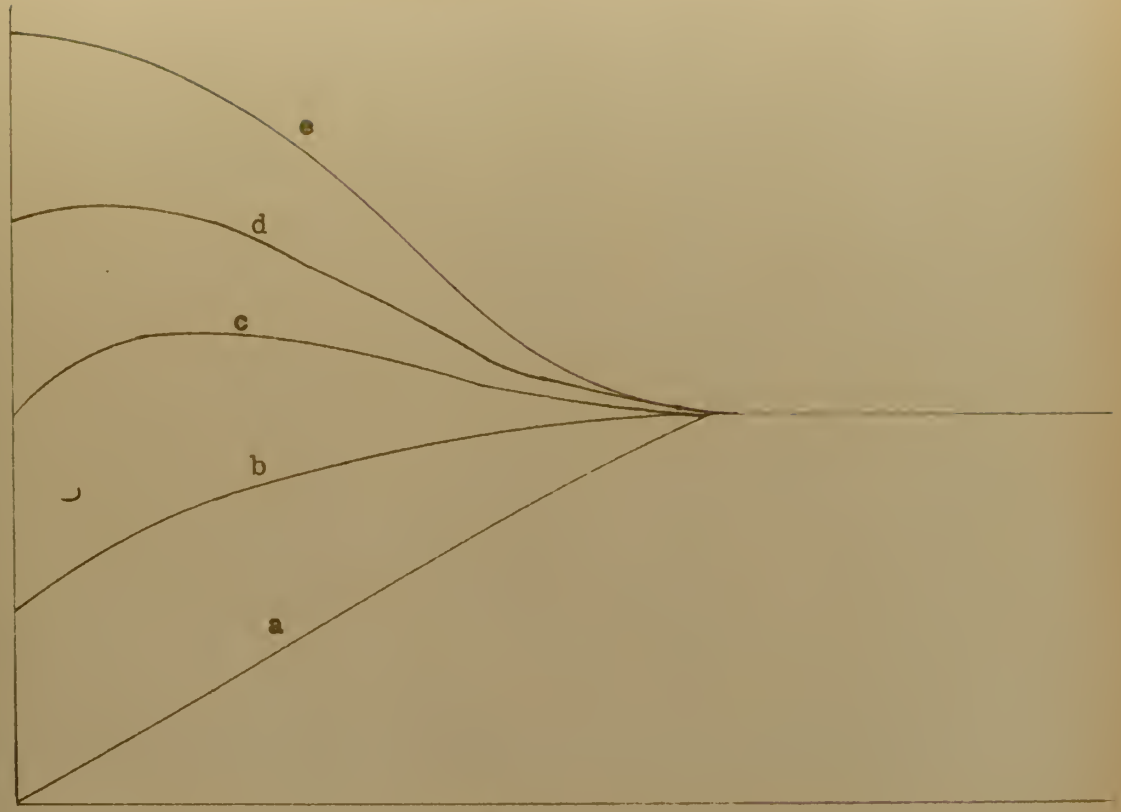


II a



II b

ILLUSTRATION II
(Part 2)



II c

Ordinate: Rate of carbon assimilation

Abscissa: Per cent air concentration

saturation (i.e., maximum rate) for photosynthesis, a curve as shown in Illustration II b would be obtained; it is seen that in the absence of air the carbon assimilation rate will be at a maximum, but that as the air concentration is increased, the molecular oxygen gradually overpowers the oxidative fragment [O] from the photosynthesis in the competition for the enzyme (E''H) which serves to reduce the oxidative fragment (and the molecular oxygen) and the curve is eventually depressed to the maximum rate of carbon assimilation for oxidative metabolism. Super-imposing these curves and the curves at intermediate illuminations (illuminations which in the absence of air give rates of carbon assimilation of one-fourth, one-half, and three-fourths the maximum respectively) the plot seen in Illustration II c is obtained. In curve c, Illustration II c, the assimilation rate of carbon with increasing per cent of air available to the bacteria at first increases, then decreases, resulting in a "hump" in the curve.

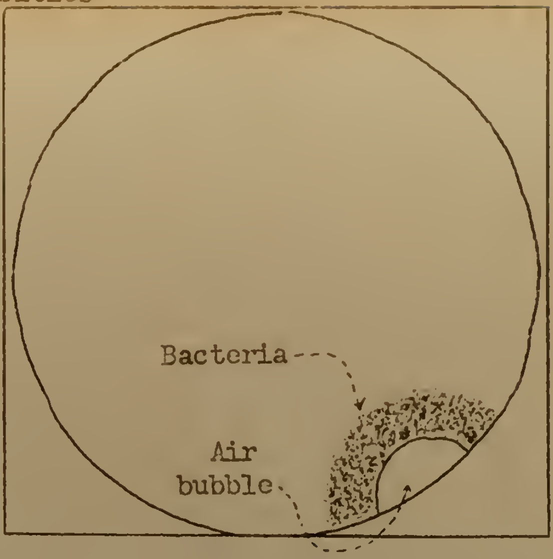
This "hump" in the curve c, (said curve representing the bacterial metabolic action under illumination of such intensity that at zero per cent air concentration the photosynthetic rate of carbon assimilation of one-half of its maximum, and equal to the maximum oxidative metabolic rate), if found will be of particular interest with respect to the Manten-Clayton hypothesis. The earlier delineation of the tactic behaviour of the Rh. rubrum was incomplete in that no mention was made of their behaviour at intermediate light intensities and oxygen concentrations. It is found that when an air bubble is introduced into a colony of these bacteria grown under a cover glass, in the dark the bacteria will cluster around the bubble and in bright light they will move away from the bubble, (exhibiting positive and

negative aerotaxis respectively). Under intermediate light intensities, however, the bacteria congregate in the narrow band at some distance from the air bubble, indicating that there is a preferred oxygen concentration between the minimum and the maximum available. These effects may be seen in Illustration V.

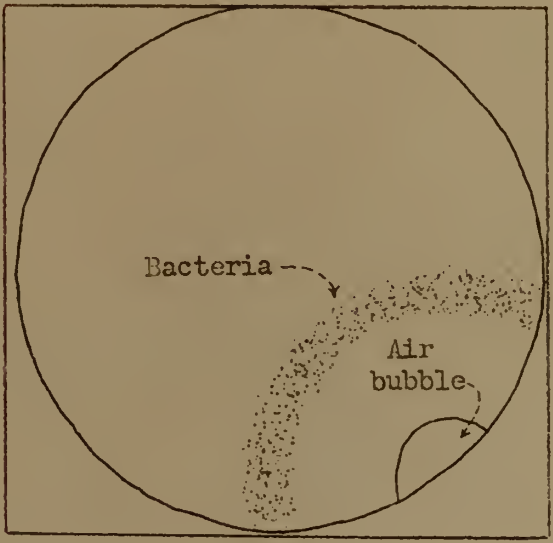
Let us now examine the curves (Illustrations I and II) in respect to their relationship to the tactic behaviour of the Rh. rubrum and the Manten-Clayton hypothesis: In the dark the metabolic rate increases with increasing oxygen concentration; this is supported by both sets of curves, and on applying the hypothesis, it would be expected that a positive aerotactic response would occur in the absence of illumination. Comparing the two curves for the metabolic behaviour under bright light, however, it is seen that the first set of curves (Illustration I) is not indicial in respect to the negative aerotaxis observed under illumination, whereas from the second set of curves (Illustration II) it can be predicted, on the basis of the Manten-Clayton hypothesis, that there will occur just such a withdrawal from areas of higher air concentrations as is observed. From the foregoing -- remembering that it is as yet largely supposition and is cited for the purpose of example -- it is strongly indicated that the tactic response is mediated through the reductive pathway -- i.e., $CO_2 \xrightarrow{[H]} (CH_2O)$ -- of the metabolism. Certainly nowhere in the first set of curves do we observe a decrease in metabolic rate with increasing air concentration such as we find in the second family. Moreover, the "hump" seen in the curve for moderate illumination very nicely explains the behaviour of the bacteria seen at moderate concentrations of air and intensities of light. To amplify this

ILLUSTRATION V

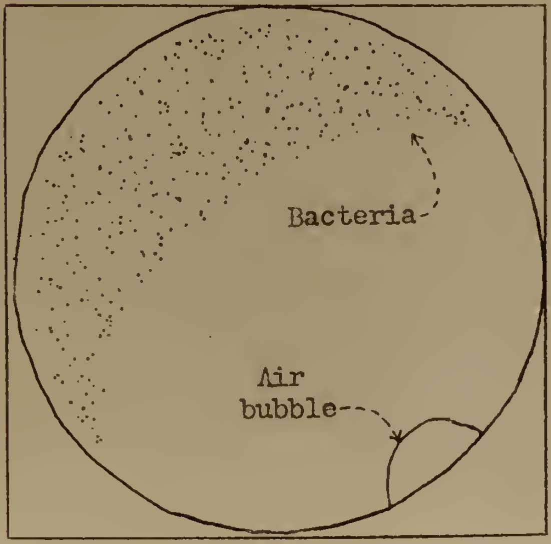
Aerotactic behaviour of Rhodospirillum rubrum under different light intensities



DARK
(V a)



MODERATE LIGHT
(V b)



BRIGHT LIGHT
(V c)

somewhat, let us imagine the Rh. rubrum in an environment of light intensity and air concentration as is exemplified by this "hump" in curve II c. Note that if the air concentration is increased or decreased there will be a decrease in metabolic rate; therefore, at this level of intensity the bacteria will congregate in the area of preferred air concentration. This is seen in Illustration V b. Moving directly upward from the "hump" to the curve II e, it can be seen that as we shift the bacterial environment to the right, i.e., into the area of increasing oxygen concentration, a decrease in the metabolic rate occurs; the contrary applies on moving to the left. This explains the behaviour actually observed, as shown in Illustration III c.

At the outset of this discussion it was pointed out that we were proceeding largely on assumptions; this being so, it may be that on analysis of the actual complete date finally obtained the evidence will not be as clear cut as the evidence from our assumptions (based in part on data already gathered). Even so, there should be enough difference between the characteristics of the curves for rate of substrate decomposition (versus per cent air) and those for the rate of carbon assimilation (versus per cent air) that a decision could be made as to which set of curves is correlated with the observed tactic responses and thereby decide with which branch of the metabolism the taxis is linked. This would constitute the first step in achieving the purpose set forth earlier.

Finally, to summarize the results of this work: It was determined that the metabolic rate of an aqueous suspension of Rhodospirillum rubrum (maintained between certain limits of hydrogen ion

concentration by addition of dilute hydrochloric acid) in the absence of light increases linearly with increasing oxygen concentration up to a maximum dependent in large measure upon the temperature of the suspension.

CHAPTER II

INSTRUMENTATION

The function of the apparatus used was to "aerate" a bacterial suspension in the absence of light with a known gas mixture at a constant flow rate, maintaining the hydrogen ion concentration between certain limits. The essential parts of the structure were: (a) a water reservoir to provide a pressure head for the gases; (b) two gas-water bottles to serve as storage and mixing chambers for the gases; (c) a flow meter to provide a method of maintaining constant flow; (d) a sintered glass funnel to contain the bacterial suspension while being "aerated", the gas entering through the pores of the sintered glass base; (e) a pH meter for the purpose of measuring changes in the hydrogen ion concentration; (f) a cover box (painted with carbon black) to exclude the light from the funnel; (g) a burette for containing the acid to be added to control the pH.

The apparatus utilized is diagrammed in Illustration III in the binder pocket; an explanation of the illustration follows:

Since the nitrogen gas from the nitrogen cylinder was determined to contain 2.8% air*, it was necessary to de-oxygenate this gas to study metabolic behaviour at yet lower air concentrations. This was done by the conventional method of passing the gas over heated copper turnings in the tubes labeled S and [illegible]. From these tubes the gas passed through the double-oblique stopcock at A, through the three-way "T" stopcock at B to the double-oblique stopcock at C, where a choice could be made to which gas-water bottle the gas would be directed. The

---

* As determined from Warburg analysis by Professor Clayton: See Appendix A

stopcock at A was for the purpose of the introduction of 100% air from a high pressure air line; the three-way stopcock at B permitted the use of the mercury manometer as a safety device in order to prevent blowing the rubber stoppers (which were wired on) from the gas-water bottles or rupturing the line at some other point. When nitrogen gas (of 2.8% air or less) was being introduced into the system, the manometer though this contamination would be in the majority of cases.

As each gas-water bottle was being filled, the one-way stopcock on the tube leading from the bottle to the 81-liter reservoir was of course open; i.e., one-way stopcock E would be open when the 14,340 milliliter capacity bottle was being filled, and similarly the one-way stopcock F would be open when the 14,400 milliliter vessel was being filled. The gas-water vessels had been volume calibrated in 500 ml. increments, except for the necks, where the calibration tape was marked in 10 and 20 ml. increments. In making the various air per cent compositions of gases, it was necessary to take into account the volume of tubing from the entrance point of the gas to the gas-water vessels. For the 14,340 ml. vessel this was 138 ml; for the 14,400 ml. vessel it was 146 ml.

The reservoir R was filled with an 0.0035 N sodium benzoate solution, the purpose of the sodium benzoate being to inhibit the formation of molds or algae in the apparatus during the course of the investigation.

When the work was first undertaken, distance from the bottom of the reservoir R to the gas water bottles was 70 centimeters. This was later increased to 138 centimeters to reduce the number of adjustments of the needle valve at G required to maintain a constant flow rate. A mathematical illustration of this is to be found in Appendix B.

The double-oblique stopcock at D permitted the selection of the vessel from which the gas was to be taken; as the gas was being drawn the corresponding stopcock (E or P) to the water reservoir R has to be open. The gas then passed on to the bubbler (or trap) at K, the primary function of which was to serve as an index to the flow rate and secondarily served as a trap between the flow-meter and the gas-water vessels. It is to be noted that the liquid volume in the bubbler at K and in the one at J is larger than is ordinarily placed in such devices. The purpose of this was to facilitate flushing of the system, as the greater the gas spaces in J and K, the greater the volume of the "new" gas that must be flushed through the system before the "old" gas was displaced.

The needle valve at G served as the only adjustment for the rate at which the gas was bubbled through the bacterial suspension, all stopcocks being either fully open or fully closed.

The gas flow metering device began with the expansion in the line between the needle valves G and H and ended with the metal "T" at I. The needle valve at H was for the purpose of adjusting the sensitivity of the flow meter; i.e., the greater the constriction at H, the greater the reading on the flow meter manometer. Once this valve was set, no necessity for changing the setting occurred. With the setting used, the flow rate could be varied from less than one-half liter/hour to more than six liters/hour. The manometer of the flow meter was filled to the proper level with water made slightly basic by the addition of sodium hydroxide, and colored by the use of phenol red indicator.

From the flow meter the gas passed through the double-oblique

stopcock at T (the purpose of which will be explained in a subsequent paragraph) to the bubbler at J. In a sense, this second bubbler was superfluous; however, early in the investigation considerable difficulty was encountered with leaks between K and F and as a consequence this bubbler at J was introduced to ascertain that the flow into the funnel at F was equal to the flow through the first bubbler at K, and that the flow meter was serving as a true indicator of the gas passing through the funnel.

For the purpose of determining the maximum rate of oxidative metabolism a break (not shown) was made in the line between J and F, and 100% air was bubbled rapidly through the bacterial suspension. The advantage of the direct use of the 100% air (rather than sending it into one of the gas-water vessels) was that it reduced the amount of time spent in flushing the system and allowed greater freedom in the schedule for mixing the gases of various percentages.

The double-oblique stopcock at T was necessitated due to the funnel's becoming clogged by the bacterial suspension while switching from the rapid bubbling by 100% air back to one of the gas mixtures contained in a gas-water bottle; this occurred even though the change was made rapidly and the tubing just below F was clamped just prior to making the change. When clogging occurred (which was readily detectable on the flow meter) the most expeditious remedy was to blow through the tubing into T thence through J and the funnel F.

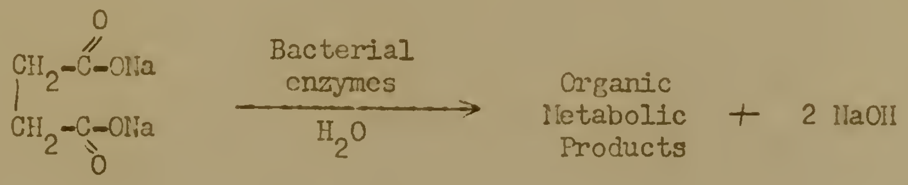
In F are seen the electrodes from the Coleman pH Meter used in these determinations. Covering F is a black box M; the light was further excluded by the careful drapping of six layers of black (focusing) cloth. At the top of M can be seen the lid through which the acid

was added into the bacterial suspension from the burette N. Although not shown, a thermometer hung near N for the purpose of checking the temperature.

CHAPTER III

PROCEDURE

The investigation consisted primarily of a series of "runs", a "run" being a procedure whereby a sequence of gases of various percentage air compositions were bubbled through a bacterial suspension, during which the oxidative metabolic rate was followed by means of the alkali production from decomposition of the substrate, using a pH meter and keeping the hydrogen ion concentration between certain limits by dropwise addition of acid. Since the substrate used was sodium succinate, the reactions may be represented as follows:



$$2\ HCl + 2\ NaOH \longrightarrow 2\ NaCl + 2\ H_2O$$

Before any "runs" were undertaken, it was necessary to standardize an acid to be used in the procedure. Both 0.0417 N HCl and 0.0100 N HCl were prepared; the latter was used for but one run, since its use resulted in excessive dilution of the bacterial suspension. Twenty five milliliters of 1.000 M sodium succinate were made to use as substrate.

A typical "run" will now be outlined:

When (after ca. 36 hours) the Rhodospirillum rubrum in the

culture medium had multiplied to the extent that 65 milliliters of the medium furnished 20 to 30 ml. of bacterial suspension of density 4.0, approximately one-half of the bacteria-infested medium was poured into fresh medium; the flask containing the fresh medium was filled to within a centimeter or two of the cotton plug to reduce the air space to a minimum, and placed in a continuously illuminated warm storage. All the transfers involving the fresh culture of bacteria were performed in flame to prevent contamination of the culture by other micro-organisms.

The remainder of the bacteria were separated from the culture medium by centrifugation and resuspended in 50 ml. of 0.0005 M phosphate buffer solution. The density of this 50 ml. suspension was determined from the bacterial densitometer (photometrically*) and the bacteria were again centrifuged and re-suspended in a volume of 0.0005 M phosphate solution such that the bacterial density was 4.0, and the bacteria were then ready for the "run". This procedure of twice suspending the bacteria in the phosphate buffer solution served to wash from the bacteria most of the nutrient material of the culture medium.

In the meantime the gas-water containers had been filled with the desired per cent air-nitrogen ratios. It had been decided to make a run of the following sequence: (a) 100% air; (b) ca. 10% air; (c) 2.8% air; (d) ??% air; (e) 2.8% air; (f) ca. 10% air; (g) 100% air; the percentage air at part (d) was to be determined by the results of parts (a), (b), and (c). (For the purposes of illustration, suppose it was desired to investigate a per cent air composition near the "saturating" per cent air; the, on a plot of ml. of acid per hour (required

*See Appendix C

to neutralize the metabolic products) versus the per cent air, the abscissa of the intersection of the line drawn through the points plotted from sequences (b) and (c) with the horizontal line through the ordinate of sequence (a) gives the "saturating" per cent. "Saturating" per cent air has been defined as the least per cent air at which oxidative metabolism occurs (as measured by substrate decomposition) at a maximum rate.)

The pH meter was standardized with buffer solution at a pH of 4.1, after which the electrodes were rinsed and placed in the funnel; the funnel had been previously moistened with distilled water. Nitrogen gas of 2.8% air was started flowing through the funnel at a slow rate (about 1 liter/hr.), and the bacterial suspension was added to the funnel. The black box was placed over the funnel and the layers of black cloth fastened around and under the box. The pH of the suspension was measured and brought to around pH 7 by the addition of acid. When conditions had become stabilized to the extent that increases in pH were merely reflecting endogenous metabolism, 1.0 M sodium succinate in the ratio of 0.03 ml. sodium succinate per ml. of bacterial suspension was added, and 100% air was bubbled through the suspension at a fast rate (ca. 8 liter/hour). The pH was reduced by the addition of acid to a value near 6.70, and the exact time (plus or minus fifteen seconds) noted that pH 6.80 was reached. The hydrogen ion concentration was allowed to decrease to around pH 7.00, whereupon the addition of acid would again increase the hydrogen ion concentration near the neighborhood of pH 6.70. (In the earlier runs the pH was allowed to vary from 6.70 to 7.20 or 7.30; however, this had the double disadvantage of introducing the factor

of operator fatigue -- one run exceeded twenty hours -- and of increasing the opportunity for an intrinsic change to occur in the bacteria which might effect their metabolic rate.) A running plot of acid added (milliliters) versus time (in minutes) was kept. The procedure was repeated three or four times -- i.e., three or four points at pH 6.80 were charted -- and gas of 10.83% air (the balance being essentially nitrogen) from one of the gas-water vessels was started bubbling through the suspension at a rate of ca. 6 liters/hour (maximum flow meter reading).

The hydrogen ion concentration was maintained between the limits pH 6.70 and pH 7.30 during the flushing process; the pH 6.80 points were charted for purposes of continuity on the graph. After 2 liters had been flushed through the suspension, the flow rate was decreased to approximately 2 liters/hour and the pH was now maintained between the limits of 6.70 and 6.90. The pH 6.80 points were again plotted, this time taking enough points to insure that the flushing process had been complete.

When sequence (b), the bubbling of the 10% air mixture through the suspension, had been completed, it was followed by the use of the 2.8% air mixture (i.e., gas originally from the nitrogen cylinder), sequence (c), flushing as before. Half way through sequence (c) the "saturating" per cent air was determined from the data so far collected to be, say, in the neighborhood of 45% air. The remainder of the 10% air mixture was exhausted through the filling port (through stopcock A; see INSTRUMENTATION) and the lines and vessel cleared by twice putting 2-4 liters of 100% air into the vessel and then

exhausting it as before. The bottle was then filled with 4,840 ml. of 100% air (allowing the volume of the gas to stabilize at the pressure due to the water head); taking into account the air in the lines, this was a total of 4,978 ml. of 100% air. The bottle was then filled with 2.8% air so that the total gas volume was 11,840 ml.; thus, a mixture of 43.67% was obtained.

At the completion of sequence (c), the effect of this 43.67% air (sequence (d)) on the oxidative metabolic rate was charted as in the previous sequences. This was followed in turn by sequences (e) (2.8% air), (f) (ca. 10% air), and (g) (100% air). Sequence (g) completed the run; the bacteria were discarded and the equipment cleaned. The symmetry of the run is to be noted, for thus were any intrinsic changes in the metabolic behaviour of the bacteria observed.

The temperature was recorded at various intervals throughout the run.

The data from an actual run is to be seen in Appendix F , and Illustration IV , the resultant plot made to the scale actually employed is one of the inserts in the pocket on the back of the binder.

The particular bacteria used in this investigation were Rhodospirillum rubrum (Strain S-1, van Niel) grown anaerobically under continuous illumination in a medium consisting of the following:

Yeast extract, 1.0%; sodium succinate, 3.0%; $(NH_4)_2SO_4$, 0.1%; $MgCl_2$, 0.05%; $CaCl_2$, 0.02%; 0.01 M NaH_2PO_4 and 0.01 M K_2HPO_4 to adjust the medium to pH 7; the balance , tapwater.

These bacteria were used in a bacterial suspension of density 4.0*

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*See Appendix C

since it was indicated early in the investigation that at this density the effects resulting from dilution (due to the addition of acid for the purpose of maintaining the pH between certain limits) were minimized, yet a workable volume of the suspension was obtained after a reasonable time from a culture not requiring an excessive amount of incubation medium.

CHAPTER IV

DISCUSSION OF RESULTS

Illustration VI is the master plot for the results obtained from the investigation, and represents five of the last six runs made from a total of thirteen (the data from the run not represented on the master plot were not discarded due to anomalies in the values -- on the contrary, the points determined from the discarded run fit in very well with the plot -- but were not used for the reason that the run involved uncontrolled variations of temperature which rendered the results untrustworthy); the earlier runs were made for the purpose of familiarizing the operator with the equipment and the behaviour of the Rhodospirillum rubrum when subjected to varying concentrations of oxygen in the complete absence of light.

It is to be noted that the curve does not pass through the origin of the plot; this can be attributed to the endogenous metabolism, which occurs at zero per cent air and involves the liberation of alkali at a rate dependent on the previous environment of the bacteria (14). The upper points are very close to the line of slope "one" (45 degree line) joining the nadir and zenith of the plot. This indicates that the "saturating" per cent air is properly defined (see PROCEDURE, page 30), and that the oxidative metabolism rate increases linearly with increasing air concentration. The variation of points falls well within the range of experimental error.

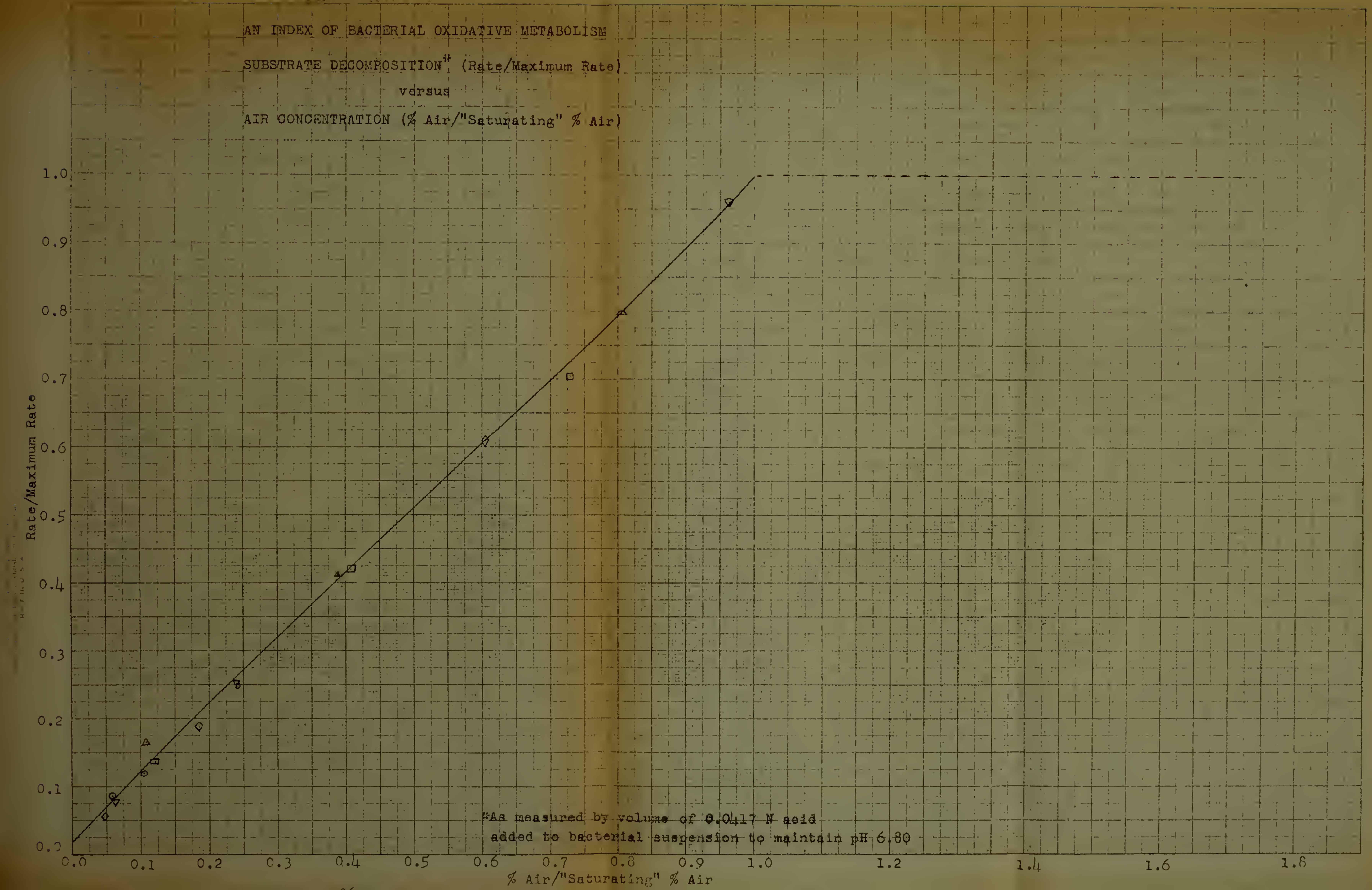
The most significant source of error was in the reading of the volume of the gas-water bottles; in the region of the 500 ml. calibration tape markings it is expected that the readings between the marks were accurate

to within only ± 30 ml.; a reading at the mark was accurate to within ± 10 ml. The maximum error in volume reading, however, would not constitute an error of serious magnitude in volumes ranging from 10,000 to 12,000 ml. Volume readings of 100% air used in mixing the various per cent air concentrations were always made on the mark, and were usually in the area where the calibration tape had been marked at 20 ml. intervals or less, and errors of ± 2 ml. were involved.

The greatest variation in the maximum metabolic rate was attributable to temperature fluctuations. Since no provision could be made for keeping the laboratory at a reasonably constant temperature, the runs were made temperally symmetrical in order to compensate for diurnal variations of temperature (see PROCEDURE, page 29).

As the volume of the bacterial suspension increased (due to the addition of acid), an increase in the efficacy of the bacterial absorption of oxygen from the gas bubbling through the suspension is to be expected, with a corresponding increase in the rate of substrate decomposition; however, this effect was probably more than countered by the loss of bacterial cells through drying around the periphery of the funnel and on the electrodes of the pH meter.

ILLUSTRATION VI



AN INDEX OF BACTERIAL OXIDATIVE METABOLISM

SUBSTRATE DECOMPOSITION* (Rate/Maximum Rate)
versus
AIR CONCENTRATION (% Air/"Saturating" % Air)

*As measured by volume of 0.0417 N acid added to bacterial suspension to maintain pH 6.80

APPENDIX A

THE WARBURG ANALYSIS

The name "Warburg Analysis" is generally used to refer to any gas analysis procedure utilizing the Warburg Apparatus, or, more properly, the Barcroft-Warburg Apparatus ("since it was adapted by Warburg from that developed by Barcroft for the study of blood gases.")(15)

This Barcroft-Warburg Apparatus consists of: (a) A suitable vessel, usually of 15 to 20 ml. capacity, with a center well and one or more side bulbs; (b) a manometer for measuring changes in gas pressure; (c) a constant temperature water bath to provide for accurate temperature control, so constructed that the manometer may be mounted on the side with the vessel containing the material to be studied completely immersed in water; and (d), a shaking device to ensure equilibrium between the fluid medium and the gas phases during the experiment which shakes the vessel and manometer horizontally at speeds of ca. 110 oscillations per minute, with an excursion of 3-4 cm.(15)

Warburg properly deserves the credit for the application of this apparatus to the study of the more intricate metabolic processes within the cell, for

> "It was Warburg who first showed that animal tissues and organs (liver, kidney, brain, etc.) could be prepared in the form of thin sections or minces which would continue to carry on metabolic processes (respiration, substrate utilization) for many hours after removal from the animal body if placed under suitable conditions, and that such metabolism could be followed quantitatively. While it is obvious that cells under these circumstances are no longer under the control of nervous or hormonal mechanisms, metabolic data

> obtained by this method of approach do not disagree with results obtained on the intact animal where the two methods can be compared, and have furnished valuable information concerning the localization of specific metabolic processes in individual organs of the animal body, as well as the effect of different substrates, coenzymes, activators, inhibitors, etc., on cell metabolism."(15)

In the investigation of Rh. rubrum metabolism, it is the living cells which are being studied, using a suspension of dead cell bodies merely as a control.

This metabolic study by the Warburg method is fraught with complexities. Carbon dioxide is assimilated -- or evolved in some cases -- oxygen is assimilated, and alkali is produced (with the consequent transfer of CO_2 from the vapor to the liquid phase). The only index of these changes, however, is a pressure change, which in turn is related to the substrate decomposition; by plotting pressure change versus time the rate of substrate decomposition can be determined. However, the pressure incorporates the three factors previously mentioned: (1) Oxygen assimilation; (2) carbon dioxide assimilation or evolvement; and (3) carbon dioxide absorbed from the gas phase into the liquid phase. To discriminate among these three it is necessary to work with pairs of vessels; the procedure may be briefly outlined as follows: (1) Into each pair of Warburg vessels containing the bacterial suspension, substrate is introduced from a side bulb; (2) acid is immediately tipped into the control vessel (also from a side bulb) which kills the bacteria, preventing any reaction from taking place and resulting in all CO_2 being driven into the gas phase; (3) in the other vessel the reaction is

allowed to go to completion, i.e., until the substrate is exhausted, charting the pressure change with time; (4) acid is tipped into the reaction vessel, releasing all of the carbon dioxide into the gas phase; and (5) alkali is introduced into both vessels from a vent, resulting in the absorption of the CO_2 into the liquid phase.

The oxygen content (expressed as per cent air) of the gases (i.e., the gas directly from the nitrogen cylinder and that which was passed over the heated copper turnings) used in the experiment was determined by absorption in basic pyrogallol in a Warburg vessel.

APPENDIX B

THE FLOW METER

It can be shown from Bernoulli's Theorem that for an incompressible fluid, neglecting friction and viscosity effects, Q (flow rate) is a function of the pressure differential between two points in the flow channel as follows:

$$P_1 + \tfrac{1}{2}rV_1^2 + gh_1r = P_2 + \tfrac{1}{2}rV_2^2 + gh_2r \qquad \text{(a)}$$

where

P : pressure

r : density of the gas

V : velocity of the gas

h_1 and h_2 : the elevation of the centers of gravity of the volumes of gas above some arbitrary datum line

g : acceleration due to gravity

Since the elevation head of a gas ($h_1 - h_2$) is generally of negligible magnitude (and in this instance, it is zero), the equation becomes

$$P_1 + \tfrac{1}{2}rV_1^2 = P_2 + \tfrac{1}{2}rV_2^2 \qquad \text{(b)}$$

or

$$\tfrac{1}{2}r(V_2^2 - V_1^2) = P_1 - P_2 \qquad \text{(c)}$$

Since the gas has been assumed to be incompressible,

and Q is the rate of flow in volume units per unit time, then $Q = A_1V_1 = A_2V_2$ where A is the area of the gas conduit. Rearranging,

$$(d) \qquad V_1 = Q/A_1 \; ; \quad V_2 = Q/A_2$$

Substituting (d) in (c), we have

$$(e) \qquad \tfrac{1}{2}r(Q^2/A_2^2 - Q^2/A_1^2) = P_1 - P_2$$

Simplifying,

$$(f) \qquad Q^2 = \frac{P_1 - P_2}{\tfrac{1}{2}r(1/A_2^2 - 1/A_1^2)}$$

$$(g) \qquad Q = \sqrt{\frac{P_1 - P_2}{\tfrac{1}{2}r(1/A_2^2 - 1/A_1^2)}}$$

Since $(1/A_2^2 - 1/A_1^2) = 1/A_2^2\,(1 - A_2^2/A_1^2)$ and letting $A_2/A_1 = n$, we have

$$(h) \qquad Q = \frac{A_2}{\sqrt{1 - n^2}}\sqrt{2/r}\,\sqrt{P_1 - P_2}$$

Designating

$$(i) \qquad K = \frac{A_2}{\sqrt{1 - n^2}}\sqrt{2/r}$$

and substituting in (h)

we have

$$(j) \qquad Q = K\sqrt{P_1 - P_2}$$

The preceding has assumed incompressibility of the fluid; for a compressible fluid, Bernoulli's equation would be

the form(23)

$$\int_{P_2}^{P_1} Q\, dP + (h_1 - h_2)mg = \tfrac{1}{2}m(V_2^2 - V_1^2)$$

Where the term $(h_1 - h_2)mg$ is zero, the equation becomes

$$\int_{P_2}^{P_1} Q\, dP = \tfrac{1}{2}m(V_2^2 - V_1^2) \qquad m: \text{ mass}$$

The expansion is essentially adiabatic, and the relationship becomes, finally,*

$$Q_1 = \frac{A_2\, \phi}{\sqrt{1 - n^2}} \sqrt{2/r}\, \sqrt{P_1 - P_2}$$

$$Q_1 = K\, \phi \sqrt{P_1 - P_2}$$

where ϕ, the compressibility factor, is

$$\phi = \frac{\sqrt{\dfrac{\left(\frac{\alpha}{\alpha-1}\right)\left[1-\left(\frac{P_2}{P_1}\right)^{\frac{\alpha-1}{\alpha}}\right]\left(\frac{P_2}{P_1}\right)^{2/\alpha}}{\dfrac{1-n^2\left(\frac{P_2}{P_1}\right)^{2/\alpha}}{1-n^2}}}}{1-\dfrac{P_2}{P_1}}$$

with α denoting the ratio C_p/C_v, i.e., the ratio of the specific heat of the gas at constant pressure to the specific heat of the gas at constant volume.

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*See page 74, reference (23)

The intermediate steps in the preceding derivation have not been presented, but can be found on pages 73 and 74 of reference (23).

For the conditions of pressure and flow, and from the dimensions of the equipment used in this investigation, it is shown in references (23)(24) and (25) that ϕ would approximate unity (ca. 0.999).

Consequently,

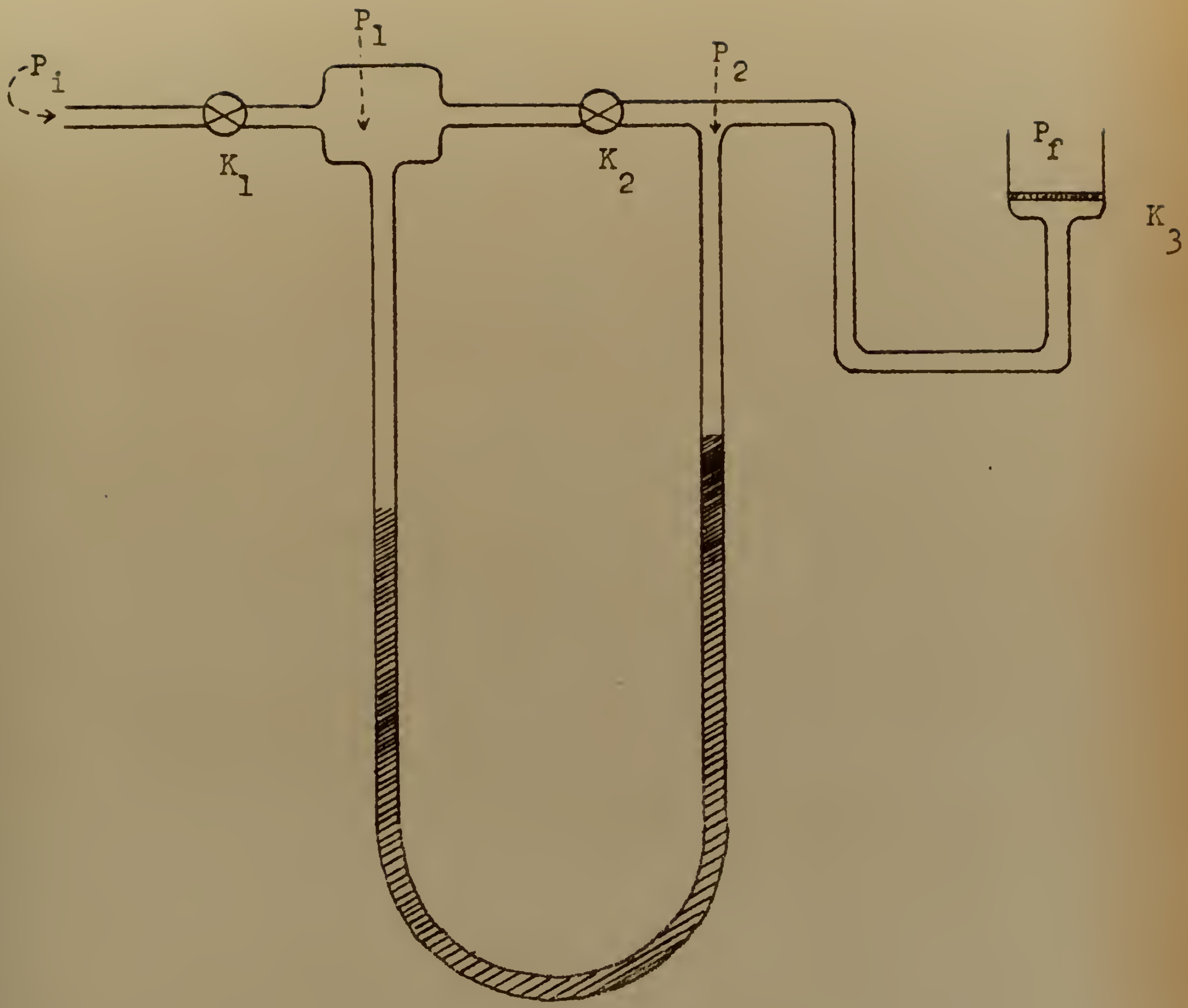
$$Q_1 = K \phi \sqrt{P_1 - P_2} \cong K \sqrt{P_1 - P_2}$$

In other words, for conditions of small flow rate and relatively small pressure differences

$$Q = K \sqrt{P_1 - P_2}$$

may be taken as the fundamental equation of flow.

This equation is utilized to show the relationships of the different resistances to flow in the flow meter used in this investigation.



P : Absolute pressure (M/T^2L)

K : Flow coefficient of pressure; varies inversely as the resistance to flow $(L^{7/2}/M^{1/2})$

Q : Flow rate; volume per unit time (L^3/T)

Q_{a-b} : Flow rate from a to b

$$Q_{i-1} = K_1\sqrt{P_i - P_1} \tag{1}$$

$$Q_{1-2} = K_2\sqrt{P_1 - P_2} \tag{2}$$

$$Q_{2-f} = K_3\sqrt{P_2 - P_f} \tag{3}$$

$$Q_{i-f} = K_{i-f}\sqrt{P_i - P_f} \quad ; \quad K_{i-f} = K \tag{4}$$

Since the flow in must be equal to the flow out,

$$Q_{i-1} = Q_{1-2} = Q_{2-f} = Q_{i-f} = Q \tag{5}$$

Then

$$K\sqrt{P_i - P_f} = K_2\sqrt{P_1 - P_2} \tag{6}$$

and

$$\sqrt{P_1 - P_2} = \sqrt{P_i - P_f}\,/(K_2/K) \tag{7}$$

Now, from (1) through (5)

$$Q^2/K_1^2 = P_i - P_1 \tag{8}$$

$$Q^2/K_2^2 = P_1 - P_2 \tag{9}$$

$$Q^2/K_3^2 = P_2 - P_f \tag{10}$$

Adding (8),(9),(10)

$$Q^2(1/K_1^2 + 1/K_2^2 + 1/K_3^2) = (P_i - P_1) + (P_1 - P_2) + (P_2 - P_f) = P_i - P_f \tag{11}$$

$$Q\sqrt{1/K_1^2 + 1/K_2^2 + 1/K_3^2} = \sqrt{P_i - P_f} \tag{12}$$

or

$$Q = \frac{\sqrt{P_i - P_f}}{\sqrt{1/K_1^2 + 1/K_2^2 + 1/K_3^2}} \tag{13}$$

Since, from (4) and (5),

$$Q = K\sqrt{P_i - P_f} \tag{14}$$

We have, then,

$$K = \frac{1}{\sqrt{1/K_1^2 \quad 1/K_2^2 \quad 1/K_3^2}} \tag{15}$$

Substituting (15) in (7), we have the relationship between the initial pressure P_i and the funnel (or final pressure P_f, and the pressures (P_1 and P_2) operating the manometer

$$(16)\quad \sqrt{P_1 - P_2} = \frac{\sqrt{P_i - P_f}}{K_2\sqrt{1/K_1^2 + 1/K_2^2 + 1/K_3^2}}$$

K and the quotient K_2/K could be determined experimentally; from these, together with the density of the manometer liquid, a numerical expression for the flow rate could be developed. For the purpose of this investigation, however, the only function of the flow meter was to enable the flow of gas to be maintained at a constant rate during a run (from 10 to 20 hours) and for this flow rate to be reproduced from one run to another.

Perhaps it should be pointed out that K_1 is a variable, and that K_3 may be subject to variation (ordinarily of a negligible magnitude). It is readily seen that, when keeping P_1, P_2, P_f, K_2, and K_3 constant, the number of adjustments of K_1 will be diminished by having P_i be of the largest magnitude practicable; i.e., since P_i will decrease slowly due to the dimunition of the water head, the consequent variation in $\sqrt{P_1 - P_2}$ (and the resultant change in manometer reading) will be lessened by having as large a P_i as possible.

APPENDIX C

THE DENSITOMETER

The bacterial densitometer employed was a simple photovoltaic photometer shown schematically in Illustration VIII.

The source was a Westinghouse 100 watt, 125 volt lamp. As in other photometers, the density is approximately the logarithm of the ratio of the meter deflections when the suspension medium (0.0005 M K_2HPO_4-NaH_2PO_4 solution) and the bacterial suspension are placed, respectively, in the beam; this is, of course, provided the source is maintained constant, that there is no error due to fatigue, and that the photocurrent is proportional to the intensity of illumination. For the uses for which this particular instrument was employed, however, it was calibrated* by utilizing successive dilutions of a known volume of bacterial cells (determined through the use of a clinical centrifuge tube). Illustration IX, the Bacterial Densitometer Chart, is the result of this calibration for the 1.5 milliampere scale on the milliammeter. The following will serve to illustrate the use of the Chart:

With distilled water in the cell, the milliammeter is set (by means of the adjusting rheostats) to read 0.35 ma (on the 1.5 ma scale). On inserting a cell filled with a portion of the 50 ml. of bacterial suspension, (suppose) the milliammeter reads 0.115, which corresponds, on referring to the chart, to a bacterial density of 1.6. Then, to have a suspension of the desired density of 4.0, the bacteria will have to be resuspended in a volume of (50)(1.6)/4 = 20 ml. of phosphate buffer.

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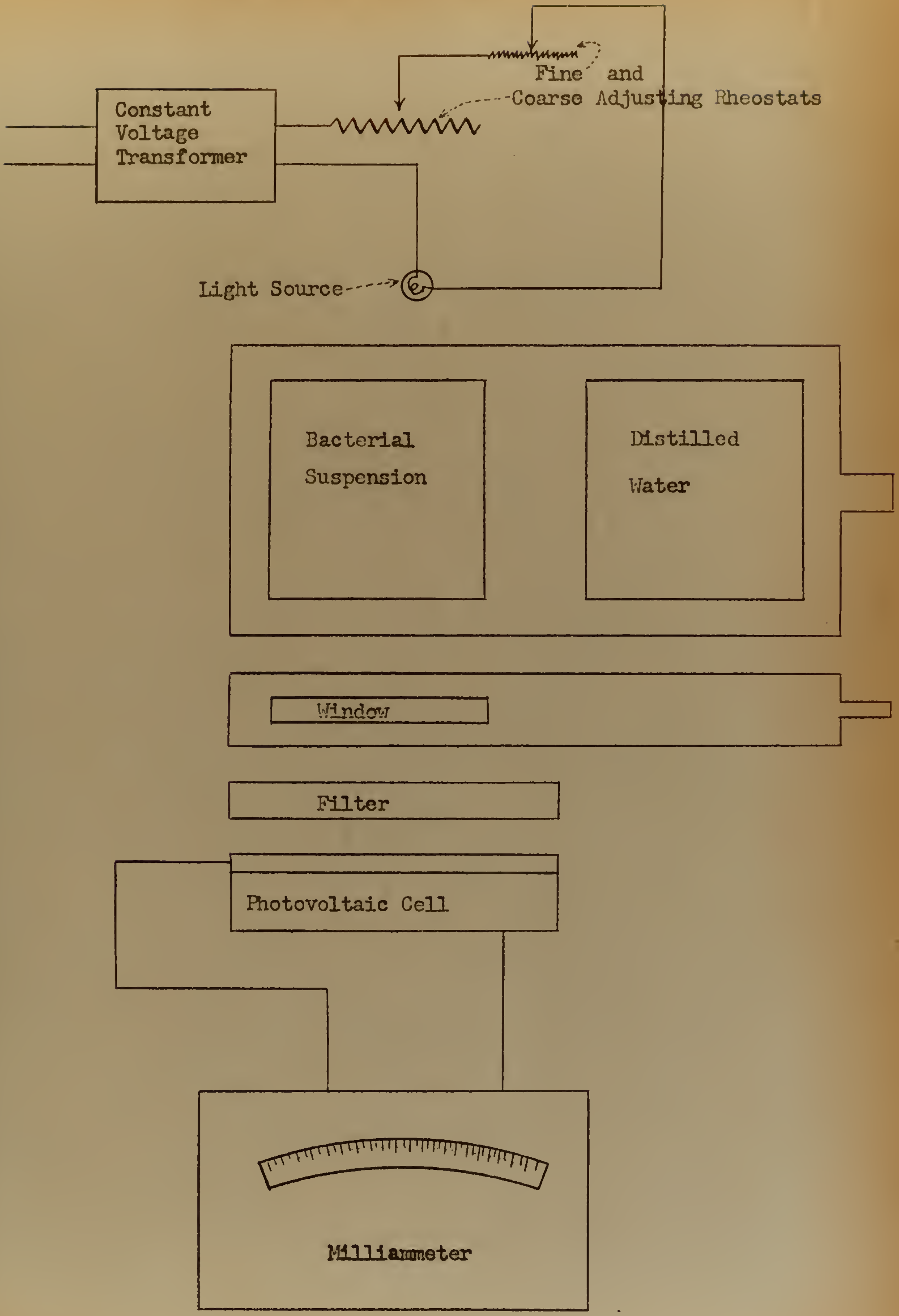
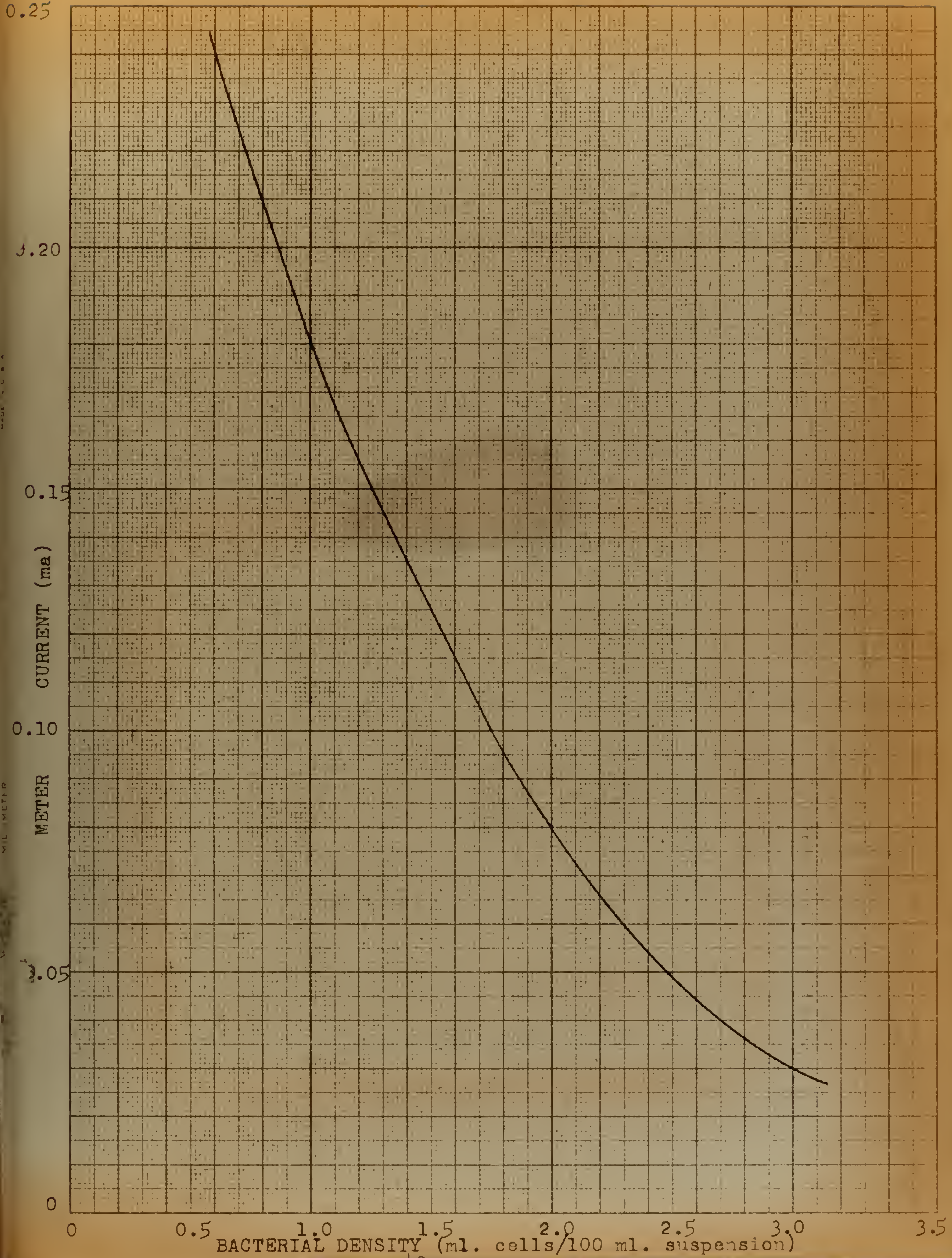
*By Dr. R. K. Clayton

ILLUSTRATION VIII

BACTERIAL DENSITOMETER



BACTERIAL DENSITOMETER CHART



APPENDIX D

THE pH METER

A Coleman OOMPAX Model 20 pH electrometer, seen in Illustration X, was employed to measure the change in pH. This is a battery operated instrument for which the manufacturer claims "a consistent sensitivity of better than 0.02 pH and consistent overall accuracy of better than 0.05 pH". It utilizes a sealed glass electrode and a reference electrode in a liquid (saturated KCl) junction reservoir. It is unique in that the pH is determined by a combination of a flashing light and a pH dial index; i.e., when the dial index is set to read the pH of the solution in which the electrodes are immersed, the indicating light will flash at the rate of once per second.

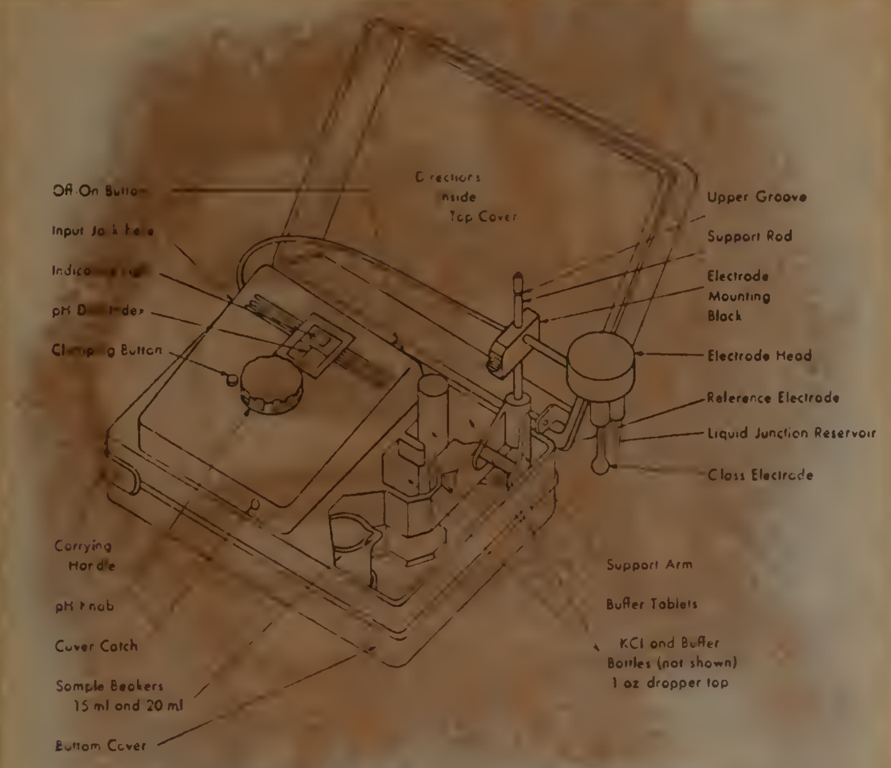


FIG. 1. MODEL 20 pH ELECTROMETER

ILLUSTRATION X

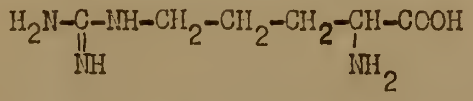
APPENDIX E

GLOSSARY OF TERMS

ACTION POTENTIAL -- A difference in potential between resting nerve and and the region of a nerve conducting an impulse.

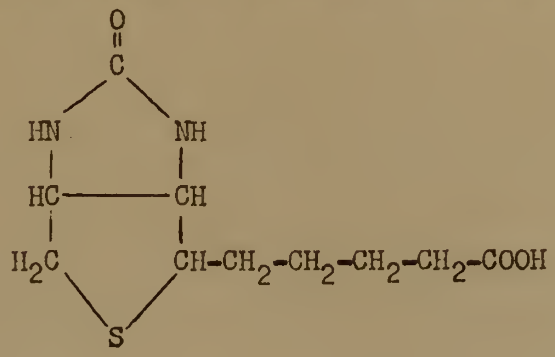
ALL-OR-NONE LAW -- If an irritable cell or unit, such as a nerve or a muscle fiber, receives a stimulus above a certain minimal (threshold) intensity, its response is maximal; increasing the stimulus results in no corresponding increase in strength of response, nor is a response elicited by a stimulus below threshold.

d-ARGININE -- α-amino-δ-guanidinevaleric acid; one of the naturally occurring diamino monocarboxylic acids, with the following structure:



$$H_2N-\underset{\underset{NH}{\|}}{C}-NH-CH_2-CH_2-CH_2-\underset{\underset{NH_2}{|}}{CH}-COOH$$

BIOTIN -- one of the B-complex vitamins, with the following structure:



BOIS-REYMOND LAW -- For a stimulus to be effective, it must be (a) above threshold intensity and (b) have reached threshold intensity before accommodation can occur.

ACCOMMODATION -- "A stimulus which reaches its full strength quickly is more effective than a slowly increasing stimulus in eliciting a response" (12); the phenomenon of an irritable system's ability to "accommodate" the slowly increasing stimulus is called accommodation.

AEROBES -- Organisms which cannot carry out life processes without free access to air.

ANAEROBES -- Facultative anaerobes are organism which can utilize atmospheric oxygen but can also grow without free oxygen; obligate (or strict) anaerobes are unable to grow in the presence of oxygen, i.e., they require that molecular oxygen be absent "and are very adversely affected by its presence".(4)

CHRONAXIE --"A term introduced by Louis Lapicque in 1909 to define the character of the stimulus which is required to excite various types of living tissue, particularly muscle and nerve. The most convenient form of stimulus is an electric current, since this can be made to excite (i.e., to cause tissue to display its characteristic activity) without doing any damage. It is found that a current must fulfil three conditions if it is to excite: (1) It must flow for more than a certain minimal duration; (2) the final strength must exceed a certain value; and (3) the rate of increase of the current from zero must exceed a definite velocity. The need for a minimal duration is shown by the fact that a current alternating at 1,000,000 cycles per second is powerless to excite any tissue in the body; and the modern surgical technique of diathermy is based on this.

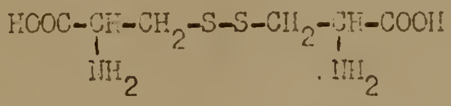
The curve relating minimal duration and minimal strength - - - as a first approximation obeys the formula of Weiss,

$$i = a + b/t$$

where *i* is the current, *t* its duration, and *a* and *b* are constants. Lapicque has shown that curves of the same form are obtained from the most diverse tissues, ranging from the human nerve, where the minimal duration is less than 0.001 second, to the cells of a plant where it may be longer than one second. Thus, the character of the stimulus for a given tissue can be defined at once if we know the constants *a* and *b* for that tissue. The constant *a* (the rheobase)) is given by the minimal current strength at long durations. The other constant *b* is the more important, for it determines the slope of the curve, it can be fixed by measuring the chronaxie, which is equal to b/a and is the least duration required when the current strength is 2a.

The true form of the curve differs somewhat from that given by Weiss's equation, and it has been used as a basis from which to deduce the mechanism of excitation. - - - the measurement of the chronaxie has also been used to investigate the passage of the state of excitation from one tissue to another (e.g., from nerve to muscle)."(26)

CYSTEINE -- di(α-amino-β-thiolpropionic acid); a naturally occurring aliphatic diamino dicarboxylic acid, with the following structure:



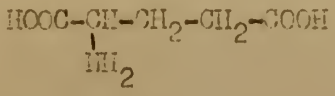
$$HOOC-\underset{NH_2}{\underset{|}{CH}}-CH_2-S-S-CH_2-\underset{NH_2}{\underset{|}{CH}}-COOH$$

ELECTROTONUS -- Physiological polarization, accomplished by passing a weak (subthreshold) constant current through a nerve; the net effect is to decrease the polarization of the membrane at the cathode, increasing the irritability (i.e., a stimulus below threshold is effective) at that point, and increasing the polarization at the anode with resultant decrease in irritability.

ENDOGENOUS -- Designating or pertaining to the metabolism of nitrogenous substances of cells and tissues, the catabolic products excreted being relatively constant in the normal organism; microbiologists define endogenous metabolism as any metabolism which takes place in the absence of an externally supplied substrate.

ENZYMES -- "Thermolabile catalysts of a definite organic nature, elaborated by living tissue but capable of action outside these tissues" (31).

d-GLUTAMIC ACID -- -aminoglutaric acid; one of the naturally occurring mono amino dicarboxylic acids, with the following structure:



$$\underset{NH_2}{\underset{|}{HOOC-CH}}-CH_2-CH_2-COOH$$

HETEROTROPHIC BACTERIA -- Bacteria which require organic carbon as a food source.

GRAM'S STAIN -- A dye of crystal violet fixed by an aqueous solution of iodine (4).

GRAM-NEGATIVE BACTERIA -- Bacteria from which Gram's stain may be washed with alcohol.

GRAM-POSITIVE BACTERIA -- Bacteria from which it is not possible to remove Gram's stain by use of common solvents (e.g., water or alcohol); that is, they dye of Gram's stain is fast in these bacteria.

LOPHOTRICATE -- Multiple polar flagellation; see frontispiece; also reference (4).

MOLAR -- A molar solution contains one gram-mole of substance dissolved in a liter of solution.

NORMAL -- A normal solution contains one gram-equivalent weight of solute in one liter of solution.

REFRACTORY PERIOD -- The period during which a muscle, nerve, or other living unit is refractory (or non-responsive or insensitive) to stimulus.

RHYTHMICITY -- Rhythmic behaviour under constant stimulation.

SUMMATION -- The phenomenon of the elicitation of a response by "the application of two or three or very few stimuli in rapid succession" although each stimuli is (a little) less than threshold strength.

WEBER-FECHNER PSYCHOPHYSICAL LAW: -- Equal increments of sensation correspond to equal increments in the logarithm of the stimulus. An interesting discussion of this "law" can be found in reference (32), and is quoted here in part (the underlining is by this writer, and not by the author of the reference):

> "Contrast-sensitivity tests are difficult to make, and the results are very erratic - - - - . The values are affected by the size and shape of the field and by the luminosity of the surroundings. They also vary with the individual and with the criterion he selects for 'barely perceptible difference.' For these and other reasons, a

different kind of test is preferable. As a result of contrast-sensitivity tests, we have the Weber-Fechner law, one of the most persistent fallacies to which erring humanity has ever committed itself. It blossoms perennially and in some quarters seems to be regarded as God's gift to the psychologist. Let us examine the evidence. The fundamental idea of this law seems to have originated in the fertile brain of Pierre Bouguer, who found, about 1760, that two surfaces could be distinguished if their contrast was 1/64. Various other investigators, including Weber (1834), checked this result with luminosities varying through a range of 10 to 1, or greater, and with c_{min} from about 1/50 to 1/150. Such values were evidently considered to be nearly constant, so that Weber's law

$$c_{min} = const$$

was adopted. This law can hardly be regarded as even an approximation. The trouble seems to be that the data were invariably plotted" - - - so - - - "the resulting curves start with high values in the scotopic region and fall almost to the axis throughout most of the photopic region. Thus it looks as if c_{min} were almost constant over a wide range of luminosities. - - - - - - - this conclusion is false," as shown by data illustrated in the reference.

- - - - - - . "It would be unwise to claim that the operational method is the 'truth' (whatever that may mean) or that it can be applied successfully everywhere -- to all mathematics, psychology, and sociology. But in physics, its success has been so marked that its use can hardly be questioned.

From the operational standpoint, most of the photometric concepts as defined in the standard definitions are meaningless. One reason for this confusion is that the concepts are complicated by a psychophysiological element which is absent in most branches of science and engineering. The psychophysiology of vision has been investigated most diligently for the past hundred years by biologists, psychologists, and physicists. Yet even the correct basis of attack has not been agreed upon. The methods of physical science have been marvelously successful in the physical world and are even applicable to such physiological problems as the action of the receptor elements in the retina and the propagation of nervous impulses. Beyond this point in the seeing process, however, we leave the world of physics and enter the world of consciousness, of sensation, in which there is no reason to believe that the methods of physics can ever be applied. We have no way of measuring sensation, in the sense that physical quantities are measured. We have no way of setting up a unit sensation -- a sensation meter stick. We have no way of applying this meter stick to determining how many times one sensation is greater than another. Consequently, there is no possibility of expression sensation mathematically as a function of stimulus. The Fechner law of logarithmic response is a mere figment of the imagination. We must realise that the difficulty of not being able to measure sensation quantitatively is not a superficial one which, by the improvement in technique and the further development of science, can in the future be eradicated. The trouble is fundamental,

> is inherent in the very fact that sensation does not reside in the world of physics and thus can never be treated as a physical quantity."

It is wondered if Mr. Moon is not a little too dogmatic in the last statement quoted.

APPENDIX F

SAMPLE RUN DATA

At start of run: Right bottle filled with 2.8% air
Left bottle filled with 10.83 % air

30.0 ml. of bacterial suspension of density 4.0

REMARKS	GAS VOLUME	FLOW RATE	ACID ADDED		TIME		pH
			Increment (milliliters)	Total	Local	Cumulative (minutes)	
Initial pH	12,800R	12			1104		7.65
Temperature 27° C.		12	1.30	1.30	1106		6.90
		12			1115		7.02
0.90 ml. 1 M sodium succinate added	12,400R	12			1117	-3	7.00
Change to 100% air	Air	Fast			1120	0	6.98
	Air	Fast	0.78	2.08(0)*	1123	3	6.80
	Air	Fast			1130	10	7.04
	Air	Fast	0.82	2.90(1)	1132.5	12.5	6.80
	Air	Fast			1137	17	7.00
	Air	Fast	0.83	3.73(2)	1140	20	6.80

REMARKS	GAS VOLUME	FLOW RATE	ACID ADDED		TIME		pH
			Increment	Total	Local	Cumulative	
	AIR	Fast			1145	25	7.00
Change to 10.83% air (after pH 6.80 reading)	Air 7,740L	Fast 46	0.84	4.57(3)	1147	27	6.80
	6,940L	46			1156	36	7.08
		46	0.91	5.48(4)	1158.5	38.5	6.80
	5,800L	46			1207	47	7.09
Change flow rate		46 12	0.88	6.36(5)	1209	49	6.80
	5,340L	12			1217	57	6.90
		12	0.44	6.80(6)	1220.5	60.5	6.80
	4,940L	12			1223.5	63.5	6.90
		12	0.38	7.18(7)	1233.5	73.5	6.80
	4,490L	12			1242.5	82.5	6.90
		12	0.43	7.61(8)	1248	88	6.80
	4,090L	12			1256	96	6.90
		12	0.40	8.01(9)	1302.5	102.5	6.80
	3,490L	12			1312.5	112.5	6.90

REMARKS	GAS VOLUME	FLOW RATE	ACID ADDED		TIME		pH
			Increment	Total	Local	Cumulative	
Change to 2.8% air	3,400L 12,400R	12 46	0.42	8.43(10)	1317.5	117.5	6.80
		46			1337	137	7.20
		46	1.03	9.46(11)	1340	140	6.80
	9,960R	46			1347	147	6.92
Change flow rate	9,280R	46 12	0.45	9.91(12)	1353.5	153.5	6.80
	8,550R	12			1414	174	6.86
	8,200R	12			1423	183	6.92
	7600 R	12	0.49	10.40(13)	1443	203	6.80
	6,830 R	12			1511	231	6.90
		12	0.30	10.70(14)	1515	235	6.80
	5,450R	12			1553	273	6.90
Change to 43.67% air	5,340R 11,340L	12 46	0.43	11.18(15)	1601	281	6.80
27° C.	10,780L	46			1613	293	7.19
		46	0.50	12.68(16)	1616	296	6.80
	10,060L	46			1619.5	299.5	7.04
Change flow rate	9,840L	46 12	1.40	14.08(17)	1622	302	6.80

REMARKS	GAS VOLUME	FLOW RATE	ACID ADDED		TIME		pH
			Increment	Total	Local	Cumulative	
		12			1623	303	7.03
		12	0.27	14.95(18)	1630.5	310.5	6.30
	9,400L	12			1634	314	6.93
		12	0.70	15.65(19)	1636.5	316.5	6.30
	9,390L	12			1639	319	6.92
		12	0.60	16.25(20)	1641.5	321.5	6.30
		12			1644	324	6.90
Change to 2.3% air	9,090L 13,000R	12 46	0.50	16.75(21)	1646	326	6.30
	12,420R	46			1650.5	330.5	7.25
		46	1.15	17.90(22)	1653	333	6.30
26.4° C.	10,990R	46			1707	347	7.20
Change flow rate		46 12	0.75	18.65(23)	1709	349	6.30
	9,950R	12			1736	376	6.90
		12	0.20	18.85(24)	1739.5	379.5	6.30
26° C.	9,950R	12			1809	409	6.92
	9,950R	12	0.32	19.17(25)	1822.5	422.5	6.30

REMARKS	GAS VOLUME	FLOW RATE	ACID ADDED Increment	ACID ADDED Total	TIME Local	TIME Cumulative	pH
	7,650R	12			1941	441	6.90
Change to 10.83% air	7,410R 11,840L	12 46	0.30	19.47(26)	1955	455	6.80
	10,800L	46			1905	465	7.22
	10,400L	46	1.00	20.47(27)	1908.5	468.5	6.80
		46			1912	472	7.00
Change flow rate	9,880L	46 12	0.61	21.08(28)	1914.5	474.5	6.80
	9,800L	12			1920	480	6.91
		12	0.30	21.38(29)	1923.5	483.5	6.80
		12			1933	493	6.92
		12	0.31	21.69(30)	1935.5	405.5	6.80
	9,090L	12			1942	502	6.90
		12	0.30	21.99(31)	1945.5	505.5	6.80
	8,790L	12			1952	512	6.90
Change to 100% air	8,590L Air	12 Fast	0.34	22.33(32)	1956.5	516.5	6.80
23° C.	Air	Fast			1959	519	7.00

REMARKS	GAS VOLUME	FLOW RATE	ACID ADDED		TIME		pH
			Increment	Total	Local	Cumulative	
	Air	Fast	0.84	23.17(33)*	2003	523	6.80
	Air	Fast			2006.5	526.5	7.00
	Air	Fast	0.61	23.73(34)	2009	529	6.80
	Air	Fast			2013	533	7.00
	Air	Fast	0.63	24.41(35)	2015.5	535.5	6.80
	Air	Fast			2019	539	7.00
	Air	Fast	0.66	25.07(36)	2022.5	542.5	6.80

*The number in the parenthesis corresponds to the points (similarly numbered) on the plot of the sample run, Illustration IV.

The changes under "REMARKS" were made after the pH meter reading of 6.80 was obtained.

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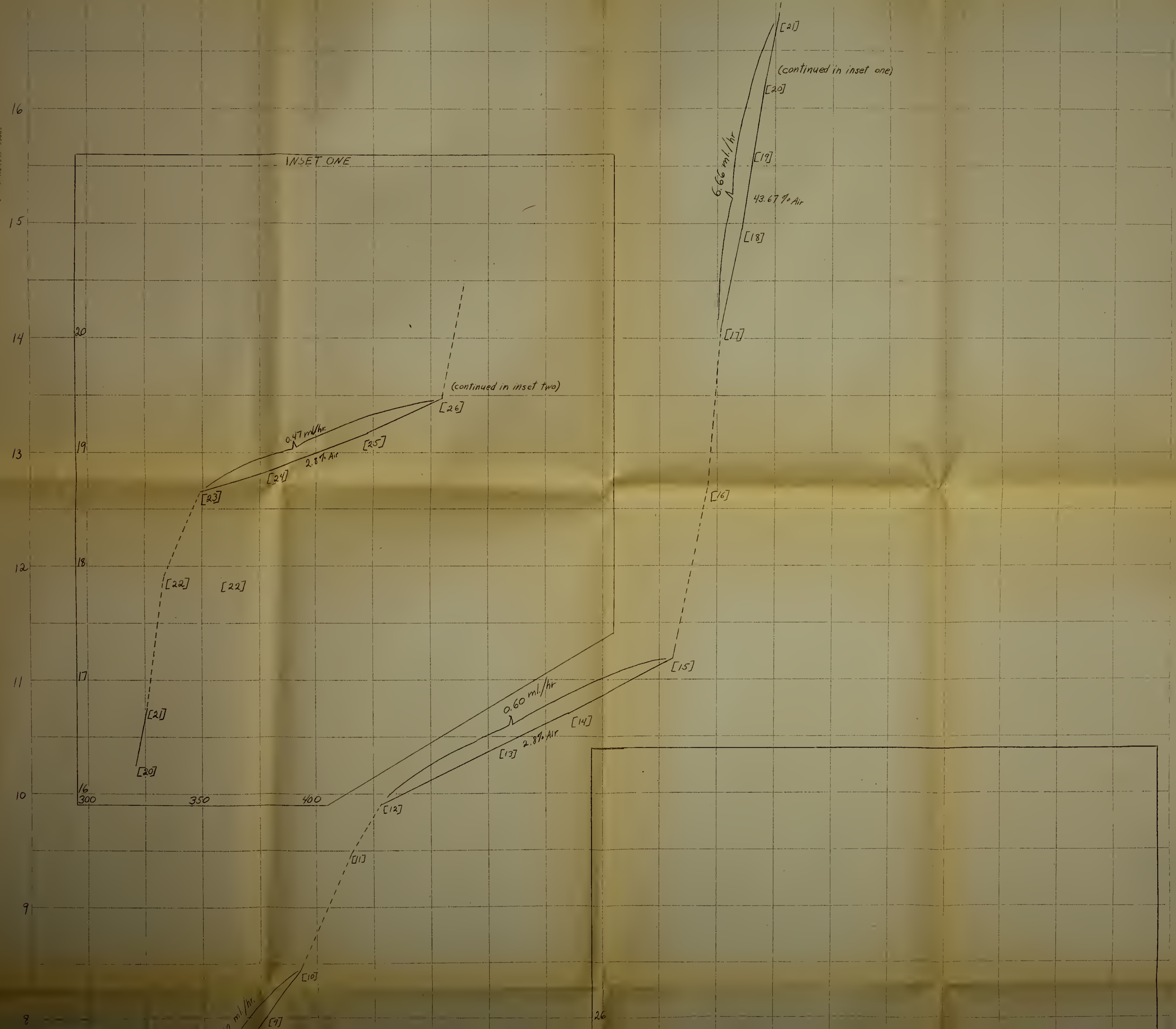
P. 45

ILLUSTRATION III


CROSS SECTION 10 x 10
16
15
14
13
12
11
10
9
8
INSET ONE
20
19
18
17
16
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400
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6.66 ml/hr
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43.67 % Air
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2.8% Air
[13]
[12]
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ml/hr
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2.8% Air
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[24]
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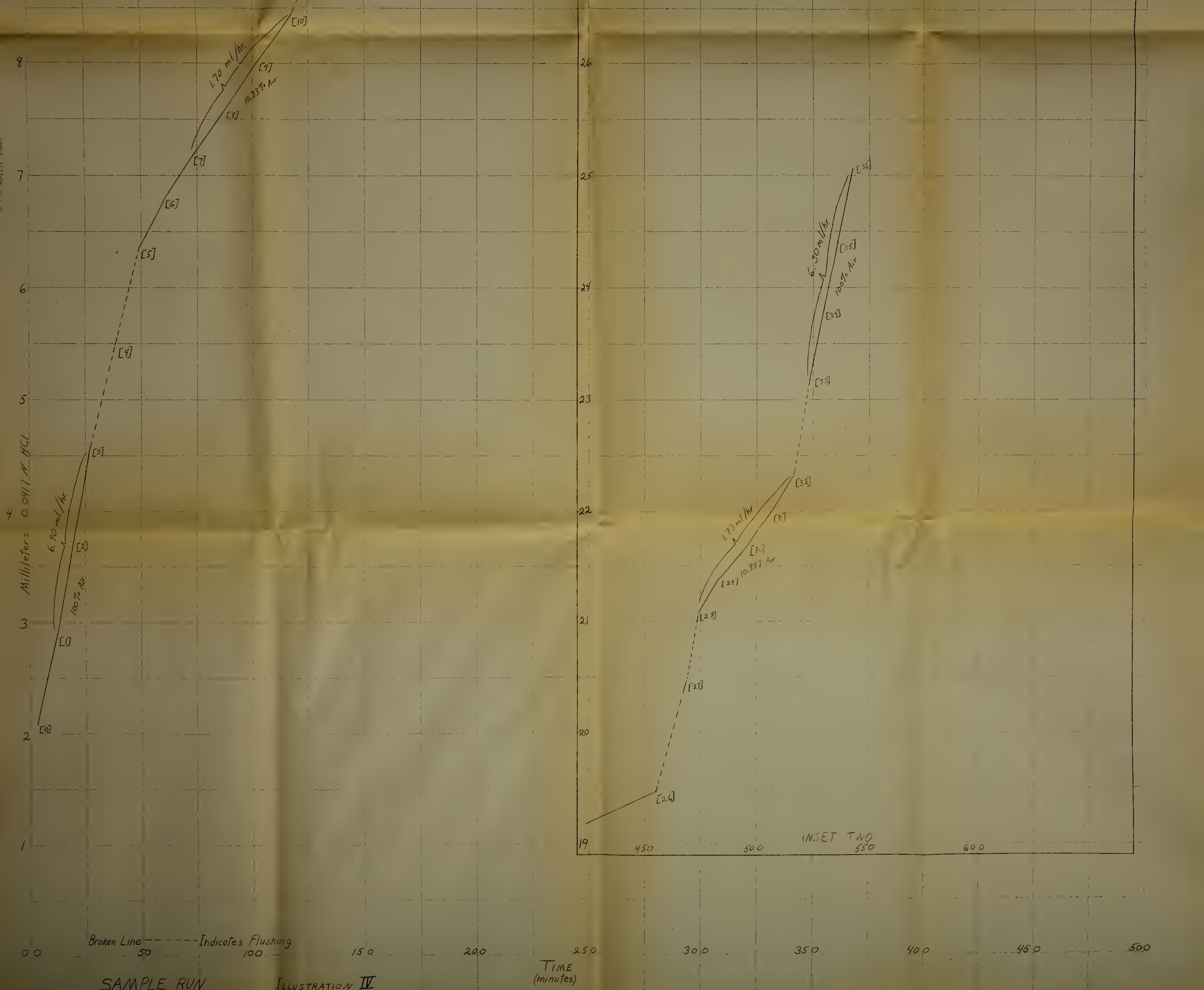
[22]
[21]
[20]
26



SAMPLE RUN

ILLUSTRATION IV

APR 1
MAY 19
MAY 18
MY 11 56

~~BINDERY~~
RECAT
DISPLAY
4578

Thesis
P45

25304

Petrie

Dependence of metabolic rate on oxygen concentration in rhodospirillum rubrum.

MAY 18
MY 11 56

BINDERY
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Thesis
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Dependence of metabolic rate on oxygen concentration in rhodospirillum rubrum.

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